THE NUCLEIC ACIDS

Volume II

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Chemistry and Biology

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Chemistry and Biology

Edited by

ERWIN CHARGAFF
Department of Biochemistry
Columbia University
New York, N. Y.

J. N. DAVIDSON
Department of Biochemistry
University of Glasgow
Glasgow, Scotland

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Contributors to Volume II

J. Brachet, Laboratoire de Morphologie Animale, Université Libre de Bruxelles, Belgium.

George Bosworth Brown, The Sloan-Kettering Institute for Cancer Research, New York, N. Y.

Alexander L. Dounce, Department of Biochemistry, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

Gertrude E. Glock, Courtauld Institute of Biochemistry, The Middlesex Hospital, London, England.

George H. Hogeboom, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

Rollin D. Hotchkiss, Rockefeller Institute for Medical Research, New York, N. Y.

I. Leslie,* Department of Biochemistry, The University of Glasgow, Glasgow, Scotland.

Peter Reichard, Karolinska Institutet, Stockholm, Sweden.

Paul M. Roll, The Sloan-Kettering Institute for Cancer Research, New York, N. Y.

F. Schlenk, Division of Biology and Medicine, Argonne National Laboratory, Lemont, Illinois.

Walter C. Schneider, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

R. M. S. Smellie, Department of Biochemistry, The University of Glasgow, Glasgow, Scotland.

Hewson Swift, Whitman Laboratory, University of Chicago, Chicago, Illinois.

B. Thorell, Department of Pathology, Karolinska Institutet, Stockholm, Sweden.

R. Vendrely, Centre de Recherches sur les Macromolécules, Strasbourg, France.

* Present address: Department of Biochemistry, The Queen's University, Belfast, Northern Ireland.

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Errata, The Nucleic Acids, Vol. II

Page 63. Reference 58 should read: gamétogénèse.
Page 65. Paragraph 2, line 6 should read: reaction.
Page 132. Heading 5 should read: Enzymes.
Page 156. Line 3 should read: shows.
Page 168. Paragraph 2, line 7 should read: Kosterlitz.
Page 169. Lines 6 and 12, and Reference 63 should read: Di Stefano.
Page 174. Line 6 should read: *Ambystoma.*
Page 176. First word should read: Her.
Page 190. Paragraph 4, line 6 should read: chromosome.
Pages 211, 218, 228, 239, and 529. For Harmon read: Harman.
Page 238. Paragraph 2, line 12 should read: hemochromogen.
Page 243. Reference 204 should read: Hogeboom.
Page 350. Paragraph 3, line 3 should read: proved to be ineffective.
Page 364. Paragraph 1, line 12, and Paragraph 2, line 9 should read: Hammarsten.
Page 512. Paragraph 2, line 7, should read: Halvorson.
Page 515. Paragraph 2, line 12 should read: unusually.
Page 518. Paragraph 2, lines 10 and 16 should read: Zamecnik.

CHAPTER 16

The Nucleic Acid Content of Tissues and Cells

I. LESLIE

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I. Introduction

Convenient and reliable methods for determining the PNA and DNA contents of tissues were not available until Schmidt and Thannhauser[1] and Schneider[2] published their procedures in 1945, and it is necessary to concentrate mainly on the large body of information accumulated since then. The situation up to 1947 has, in any case, been thoroughly reviewed and tables of data provided by Davidson and Waymouth[3] and Davidson.[4-6]

[1] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.*, **161**, 83 (1945).
[2] W. C. Schneider, *J. Biol. Chem.* **161**, 293 (1945).
[3] J. N. Davidson and C. Waymouth, *Nutrition Abstr. & Revs.* **14**, 1 (1944–1945).
[4] J. N. Davidson, *Symposia Soc. Exptl. Biol.* **1**, 77 (1947).
[5] J. N. Davidson, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 50 (1947).
[6] J. N. Davidson, "The Biochemistry of the Nucleic Acids," 2nd ed. Methuen, London, 1953.

New methods of expressing results have come into use in recent years. Since Boivin, *et al.*[7] and subsequent investigators established that there is a constant average amount of DNA per nucleus for any animal tissues (Chapter 19), DNA has become a useful standard of reference for revealing the changes in cell number and cell composition and has been used in this capacity by a number of authors.[8-12] In cases where it has been possible to compare results as expressed in amount per organ, amount per average cell, and concentration per unit weight of tissue, it has become clear that the last method of expression, which is the one most commonly employed, can, in certain circumstances, be completely misleading.[13, 14]

However, it is essential to study various aspects of tissue composition simultaneously. In this review an attempt has been made to obtain as far as possible from published results the amounts of PNA and DNA in terms of content per organ, concentration per unit of fresh weight, concentration per unit of protein nitrogen, amount per average cell, and the ratio PNA/DNA.

It has been disappointing to find so little information on the direct quantitative relationship between nucleic acids and protein in tissues in view of the widely accepted association between these components. Information about the content of nucleic acids in tissues is, of course, insufficient evidence on which to come to a final conclusion about their function. To illustrate the difficulties, one need only mention the finding of Munro *et al.*[15] that, whereas an increase in energy intake would not increase the content of liver PNA on a protein-free diet, it significantly raised the uptake of P^{32} by PNA.

II. Methods

Determinations of the nucleic acids are usually based on (1) their phosphorus content, after separation of PNA and DNA, (2) the pentose of PNA and the deoxypentose of DNA (see Chapter 9), or (3) the ultraviolet absorption in the region 357 to 270 mμ of the purine and pyrimidine components (see Chapter 14). The last two methods are more specific than the first, but it is important to select suitable samples of PNA and DNA as standards, since the proportions of purines to pyrimidines vary according

[7] A. Boivin, R. Vendrely, and C. Vendrely, *Compt. rend.* **226,** 1061 (1948).
[8] R. Bieth, P. Mandel, and R. Stoll, *Compt. rend. soc. biol.* **142,** 1020 (1948).
[9] J. N. Davidson and I. Leslie, *Nature* **165,** 49 (1950).
[10] J. N. Davidson and I. Leslie, *Cancer Research* **10,** 587 (1950).
[11] R. M. Campbell and H. W. Kosterlitz, *J. Endocrinol.* **6,** 308 (1950).
[12] J. M. Price and A. K. Laird, *Cancer Research* **10,** 650 (1950).
[13] I. Leslie and J. N. Davidson, *Biochim. et. Biophys. Acta* **7,** 413 (1951).
[14] R. Y. Thomson, F. C. Heagy, W. C. Hutchison, and J. N. Davidson, *Biochem. J.* **53,** 460 (1953).
[15] H. N. Munro, D. J. Naismith, and T. W. Wikramanayake, *Biochem. J.* **54,** 198 (1953).

to the source of the nucleic acids.[16] In estimations of the sugar components, the sugars of the purine nucleotides are mainly responsible for the intensity of coloration. The ultraviolet absorbing properties of a nucleic acid also depend to some extent on the proportions of purine and pyrimidines. Whichever of these two methods is employed, the procedure is simplified if the results are expressed in terms of the P content of the standard PNA or DNA. The average amount of P in PNA is 9.4 % as compared with the 9.9 % for DNA.[1]

Until 1945, the nucleic acids were estimated largely as total nucleic acid phosphorus (NAP)[17] or on the basis of their purine content.[18, 19] Because PNA and DNA differ in their location within the cell and in their physiological behavior, information about the total nucleic acid content is in most cases not very illuminating.

When PNA is determined by the color reaction of its pentose with orcinol[20] and DNA by the reaction of deoxypentose with diphenylamine,[21] substantial errors may arise if there is incomplete separation of the nucleic acids from interfering tissue constituents, such as glucose, glycogen, mucopolysaccharides, proteins, or amino acids. Davidson and Waymouth[22] and von Euler and Hahn[23] tried to avoid such sources of error by extracting the nucleic acids from tissue residues with sodium chloride and dilute alkaline solutions and precipitating them as their lanthanum salts. But this procedure, apart from being tedious, is subject to errors[5] and has been superseded by more convenient methods.

The two most commonly employed are those of Schmidt and Thannhauser[1] and of Schneider.[2] The more recent method of Ogur and Rosen[24] appears to be of more limited application. The various fractions obtained in these procedures are shown in Table I. All three methods involve a preliminary extraction of acid-soluble compounds (fractions I and XIII) and lipids (II, XI, and XII).

1. Procedure of Schmidt and Thannhauser (1945)

In the Schmidt and Thannhauser[1] method the PNA is separated from DNA and most of the tissue protein, after overnight incubation at 37° with normal alkali. Acidification of the alkaline digest III with trichloroacetic

[16] E. Chargaff, Chapter 10, and B. Magasanik, Chapter 11 in this book.
[17] I. Berenblum, E. Chain, and N. G. Heatley, *Biochem. J.* **33**, 68 (1939).
[18] N. Alders, *Biochem. Z.* **18**, 400 (1927).
[19] T. B. Robertson and M. C. Dawbarn, *Australian J. Exptl. Biol. Med. Sci.* **6**, 261 (1929).
[20] W. Mejbaum, *Z. physiol. Chem.* **258**, 117 (1939).
[21] Z. Dische, *Mikrochemie* **8**, 4 (1930).
[22] J. N. Davidson and C. Waymouth, *Biochem. J.* **38**, 39 (1944).
[23] H. von Euler and L. Hahn, *Svensk Kem. Tidskr.* **58**, 251 (1946).
[24] M. Ogur and G. Rosen, *Arch. Biochem.* **25**, 262 (1950).

TABLE I

METHODS FOR DETERMINING PNA AND DNA

Fraction	Description of procedure	Phosphorus compounds in fraction
	1. Procedure of Schmidt and Thannhauser[1]	
Fresh tissue	Should be used immediately or stored at −10°. All volumes should be at least 20 times that of samples, and tissue should be homogenized in ice-cold water.	
I	Ice-cold 5–10% trichloroacetic acid (TCA) or perchloric acid (PCA) extract. Extraction to be carried out rapidly and repeated three times.	All acid-soluble compounds
II	Combined extracts of tissue residue by 80% ethanol, ethanol, warm ethanol-chloroform (3:1), or ethanol-ether (1:1) and ether.	Phospholipids
III	Alkaline digest formed by overnight incubation in *N* NaOH or KOH at 37° of tissue residue from II (1 ml. alkali per 100 mg. fresh tissue).	PNA-P + DNA-P + concomitant (non-nucleotide) P
IV	Supernatant formed by addition to III of HCl (to neutralize NaOH) and TCA or PCA to give final concentration of 5–10%. Precipitate centrifuged down at 0° and washed twice with 5% TCA or PCA.	PNA-P + concomitant P
V	Precipitate of inorganic PO_4^{---} from IV by method of Delory[25] or Mathison.[26]	"Phosphoprotein" or part of concomitant P
VI	Precipitate from TCA or PCA treatment of III.	DNA-P (+ protein)
VII	Extract of VI with 5% TCA at 90° for 15 min.—two washings with 5% TCA.	DNA for ultraviolet absorption[28]
	2. Procedure of Schneider[2]	
Fresh tissue	As for procedure 1.	
I	As for procedure 1.	Acid-soluble compounds
II	As for procedure 1.	Phospholipid
VIII	Tissue residue.	PNA-P + DNA-P + concomitant
IX	Extract of VIII with 5% TCA or 6% PCA at 90° for 15 min. Combined with two washings with acid.	PNA-P + DNA-P + some concomitant P
X	Insoluble residue after acid extraction of VIII.	"Phosphoprotein," P, and residual concomitant P
	3. Procedure of Ogur and Rosen[24]	
Fresh tissue	Kept in 70–95% ethanol at 0°, and homogenized prior to extraction.	
XI	Extract of homogenate with 70% ethanol at 4°, washed again with 70% ethanol containing 0.1% PCA at 4°.	Alcohol-soluble compounds
XII	Extract of tissue residue with boiling ethanol ether (3:1). Repeated, and extracts combined.	Alcohol-ether soluble compounds
XIII	Cold, rapid extraction of tissue residue from XII with 0.2 *N* PCA. Repeated, and extracts combined.	Acid-soluble compounds
XIV	Tissue residue from XIII.	PNA-P + DNA-P + concomitant P
XV	Extract of XIV with *N* PCA for 18 hr. at 4°. Two washings with *N* PCA. Extracts combined.	PNA-P
XVI	Extract of tissue residue from XV with 0.5 *N* PCA at 70° for 20 min. Process repeated, and extracts combined.	DNA-P + concomitant P

acid (TCA) or perchloric acid (PCA) allows the DNA and protein to be centrifuged down (VI) while the PNA remains in solution as acid-soluble nucleotides (IV). The two nucleic acids can be determined on the basis of the P content of IV and VI, if the amount of "phosphoprotein" in the former is small enough to be neglected.[1] A closer estimate of PNA is obtained by precipitating the inorganic phosphate from "phosphoprotein" in IV[25, 26]; PNA phosphorus (PNA-P) is represented by the difference (IV–V).

For rat liver tissue nonnucleotide phosphorus derivatives in IV are now known to represent about 25 % of the total P of the fraction.[27] High amounts of nonnucleotide P have been found by Logan *et al.*[28] in the same fraction from brain tissue; these varied from 66 % of the total NAP in white matter to 37.5 % in gray matter. Fraction IV from bacterial and yeast cells also contains relatively large amounts of nonnucleotide P.[29-31] In *Streptococcus faecalis* most of the DNA remains undissolved during the alkaline incubation.[31]

Various modifications of the Schmidt and Thannhauser procedure have been introduced. Schmidt *et al.*[32] used 1% egg albumin to produce a flocculent precipitate (VI), since the relatively small amounts of DNA in *Arbacia* eggs were otherwise difficult to precipitate after acidification of III. Davidson *et al.*[33] adapted the procedure to the very small amounts of PNA and DNA in tissue explants growing *in vitro*, and Scott and Fraccastoro[34] report a modification suitable for micro-amounts in tissue sections. Where digestions of fractions IV and VI are carried out with concentrated PCA at 210°, NaOH has to be used instead of KOH for the alkaline digest in order to avoid formation of insoluble potassium perchlorate. If PNA is to be measured by ultraviolet absorption, PCA is a more convenient precipitating agent than TCA because of its low optical density in the wavelength region 257 to 270 mμ. If necessary, it can subsequently be removed as potassium salt, as Davidson and Smellie[35] have done in order to obtain solutions of nucleotides suitable for treatment by paper inophoresis. This method enables the PNA-P to be determined from the summation of the P content of its component nucleotides, thus eliminating errors due to the concomitant P of fraction IV.

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[25] G. E. Delory, *Biochem. J.* **23,** 1161 (1938).
[26] G. C. Mathison, *Biochem. J.* **4,** 233 (1909).
[27] J. N. Davidson and R. M. S. Smellie, *Biochem. J.* **52,** 599 (1952).
[28] J. E. Logan, W. A. Mannell, and R. J. Rossiter, *Biochem. J.* **51,** 470 (1952).
[29] P. Mitchell and J. Moyle, *J. Gen. Microbiol.* **5,** 966 (1951).
[30] J. M. Wiame, *J. Biol. Chem.* **178,** 919 (1949).
[31] H. S. A. Sherrat and A. J. Thomas, *J. Gen. Microbiol.* **8,** 217 (1953).
[32] G. Schmidt, L. Hecht, and S. J. Thannhauser, *J. Gen. Physiol.* **31,** 203 (1948).
[33] J. N. Davidson, I. Leslie, and C. Waymouth, *Biochem. J.* **44,** 5 (1949).
[34] J. F. Scott and A. P. Fraccastoro, *J. Natl. Cancer Inst.* **13,** 203 (1948).
[35] J, N. Davidson and R. M. S. Smellie, *Biochem. J.* **52,** 594 (1952).

2. Procedure of Schneider (1945)

In the Schneider[2] procedure (Table I) the PNA and DNA are extracted together by means of hot (90°) TCA or PCA,[36] thus freeing them from most of the tissue protein which remains insoluble (X). As both nucleic acids are hydrolyzed to their constituent nucleotides or free bases (IX), there is no means of separating them. Instead they are determined on the basis of specific color reactions for pentose and deoxypentose.

Schneider[37] carried out an extensive series of parallel determinations on animal tissue using his own and the Schmidt and Thannhauser procedures, and found reasonably good agreement between the two methods. According to McIndoe and Davidson,[38] the Schneider treatment splits off from the protein residue all the reactive sugar components, leaving appreciable amounts of P bound to the protein residue (X). If the extraction is made with hot PCA, there is the danger of appreciable contamination of the extract by protein and its breakdown products.[28]

Logan *et al.*[28] also report good agreement between the Schmidt and Thannhauser and the Schneider procedures as applied to pancreas, spleen, thymus, white blood cells, and reticulocytes, although wide discrepancies are found between parallel determinations on brain or nerve tissue. Phosphoprotein, an inositide-P complex, and unspecified chromogenic materials are apparently present in substantial amounts in nerve tissue and interfere with the determinations. In these circumstances a combination of the two procedures involving ultraviolet absorption measurements of fractions VII and IX proved the most reliable means of determining PNA and DNA. The amount of DNA could be obtained directly from fraction VII. A duplicate sample of tissue was put through the Schneider procedure and PNA determined from the difference (IX–VII). The Schneider procedure gave very high values for PNA (orcinol method) when applied to serum[39]; on the other hand, for samples of yeast cells it gave lower values for PNA than those obtained by the Schmidt and Thannhauser procedure.[40] Ogur *et al.*[41] report the extraction of total nucleic acid and metaphosphate from yeast residues by three extractions with 5% PCA at 70°.

Two micro-modifications of the Schneider procedure have been developed. The method of Steele *et al.*[42] requires special micro-equipment and covers the range of 9 to 58 μg. PNA and 1.6 to 15 μg. DNA. Patterson and Dackermann[43] employed the micro-equipment of Linderstrøm-Lang and Holter[44] and measured the nucleic acids of *Drosophila* salivary glands in amounts of 1 to 2 μg. DNA and 10 to 20 μg. PNA.

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[36] W. C. Schneider, G. H. Hogeboom, and H. E. Ross, *J. Natl. Cancer Inst.* **10,** 977 (1950).

[37] W. C. Schneider, *J. Biol. Chem.* **164,** 747 (1946).

[38] W. M. McIndoe and J. N. Davidson, *Brit. J. Cancer* **6,** 200 (1952).

[39] R. H. Common, D. G. Chapman, and W. A. Maw, *Can. J. Zool.* **29,** 265 (1951).

[40] A. Bourdet, P. Mandel, and R. Guillemet, *Compt. rend.* **232,** 756 (1951).

[41] M. Ogur, S. Minckler, G. Lindegren, and C. C. Lindegren, *Arch. Biochem. and Biophys.* **40,** 175 (1952).

[42] R. Steele, T. Sfortunato, and L. Ottolenghi, *J. Biol. Chem.* **177,** 231 (1949).

[43] E. K. Patterson and M. E. Dackermann, *Arch. Biochem. and Biophys.* **36,** 97 (1952).

[44] K. Linderstrøm-Lang and H. Holter, *in* "Die Methoden der Fermentforschung" (E. Bamann and K. Myrbäck, eds.), p. 1132. Thieme, Leipzig, 1940.

3. Procedure of Ogur and Rosen (1950)

This method[24] (Table I) was developed to deal with the problem of determining PNA and DNA in amounts as low as 1 μg. in plant root tips and pollen cells. Because of the small amounts of material available and the presence of interfering substances (pentosans, polyuronides), both the Schmidt and Thannhauser and the Schneider procedures were considered inadequate for the purpose.

The preliminary extraction of the tissue begins with alcohol treatment (XI and XII) in order to eliminate as soon as possible an alcohol-soluble compound which gives an intense purple coloration with diphenylamine. The overnight treatment of tissue residue XIV at 4° with *N* PCA extracts only PNA, but repeated extractions may be necessary when the PNA/DNA ratio is high. In this fraction (XV) PNA is measured by ultraviolet absorption at 260 mμ or on the basis of its P content.

The DNA is subsequently extracted from the tissue residue by hot acid treatment (XVI) and can be determined by the diphenylamine reaction,[21] ultraviolet absorption at 270mμ, or the P content of the fraction. In plant material, about 3 % of the total P remains in the final residue.

The method is not equally effective with all tissues. Patterson and Dackermann[43] could get only partial extraction of PNA from *Drosophila* salivary glands, and Ogur *et al.*[41] report that in yeast cells part of the DNA is extracted during cold PCA treatment. Koenig and Stahlecker[45] applied a modified procedure to fixed tissue sections from mammalian liver and nerve tissue. In certain species of bacteria 30 hrs. of exposure to PCA was needed to remove all the PNA.[46]

III. Normal Adult Tissues

Very often in the literature the terms "content" and "concentration" are employed as synonyms, when, in fact, they should refer to two distinct features. In this review the term "content" will be used only when results are given as amounts per organ or as amounts per cell. The term "concentration" will be confined to describing amounts per unit weight of tissue or per unit of protein nitrogen.

Information on the amounts of PNA-P and DNA-P in normal adult tissues is collected in Table II (rat tissues) and Table III (other animals and man). More data are available for the rat than for any other animal, and the average amounts of DNA-P per nucleus have been carefully determined in a number of adult rat tissues.[14] Owing to the high proportion of polyploid nuclei in rat liver, the average amount of DNA-P per nucleus is higher in liver than in any other tissue (Table II). This point is discussed in detail in Chapter 19.

[45] H. Koenig and H. Stahlecker, *J. Natl. Cancer Inst.* **12,** 237 (1951).
[46] W. A. Cassel, *J. Bacteriol.* **59,** 185 (1950).

TABLE II

AMOUNTS OF RIBONUCLEIC ACID PHOSPHORUS (PNA-P) AND DEOXYRIBONUCLEIC ACID PHOSPHORUS (DNA-P) IN ADULT RAT TISSUES[a]

Tissue	Weight of organ, g.	Total per organ		Micrograms per 100 mg. fresh tissue		Micrograms per mg. protein N		Picograms per average cell[b]		Ratio PNA-P / DNA-P	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Liver	7.67	7.19	1.66	93.4	21.6	36.9	8.6	4.0	0.913	4.38	(14)
				95	24					3.95	(1)
	12.0	9.21	2.02	77.5	16.8	33.5	7.25	5.25	1.14	4.62	(47)
	5.46	5.56	1.07	102	19.7	36.3	7.0			5.14	(48)
						34.8	10.6			3.28	(49)
Pancreas				198	48				0.712	4.1	(14, 37)
				178	45.2					3.96	(50)
Testis				41	23					1.78	(51)
Seminal vesicle		0.107	0.056	48.5	25.4					1.94	(52)
Small intestine				73.2	129.2				0.738	0.57	(14, 23)
Brain	1.52	0.56	0.21	36.8	13.8	25.4	9.2			2.67	48
				18.8	20.0					0.94	(37)
				13.5	9.4					1.43	(53)
				17.5	12.3					1.42	(50)
Lung				52.0	92.1				0.651	0.57	(14, 23)
				18.0	60.5					0.30	(50)
				36.0	71.0					0.5	(54)
Kidney[c]	0.73	0.480	0.195	65.7	26.7	27.3	11.3		0.652	2.46	(14, 48)
				40.7	41.8					0.97	(37)
				29.0	32.4					0.89	(55)
	0.694	0.484	0.213	69.7	30.7	34.3	15.1			2.27	(56)
Heart				31.4	30.6				0.627	1.03	(14, 57)
				12.4	14.5					0.85	(50)
Skeletal muscle[d]				6.7	5.7					1.17	(50)
					4.3						(58)
				20	9.6					2.1	(53)
	7.41	1.80	0.37	24.5	5.0	38.6	7.9			4.9	(59)
Thymus				53	276				0.718	0.19	(14, 37)
				38	264					0.14	(50)
Spleen[c]				49.9	140				0.633	0.36	(14, 37)
	0.52	0.40	0.40	77	77	36.4	36.4			1.0	(60)
				86.4	84.1					1.03	(61)
				58	165					0.35	(54)
Bone marrow				87	153				0.670	0.57	(14, 51)
				126	130					0.97	(61)

[a] Many authors express their results as amounts of PNA or DNA. For the purpose of this and the following tables, their figures have been divided by 10, on the basis of the assumption that the P content of both PNA and DNA is approximately 10%.

[b] 1 picogram (pg.) = 10^{-12} gram.

[c] There is a distinct lack of agreement among the results of various authors for kidney and spleen (Table II). The explanation seems to lie in the different methods employed to measure PNA and DNA. When the Schmidt and Thannhauser[1] method is used and PNA determined by measuring the P content of fraction IV (Table I) the ratio PNA-P/DNA-P is over 2 for kidney and 1 for spleen (48, 56, 60, 61). When the Schneider method (2) is employed, the PNA measured by orcinol and DNA by diphenylamine, the PNA-P/DNA-P ratio is less than 1 for kidney (37, 55) and about 0.3 for spleen (37, 54). In kidney, the difference must lie in the determination of PNA, as the DNA concentrations are much the same, whatever the method employed. But, in spleen, the Schneider method gives a much higher figure for DNA and a lower value for PNA than the Schmidt and Thannhauser method. The most likely errors in the former method arise from interference by chromogenic substances in the orcinol and diphenylamine procedures, whereas in the latter procedure the PNA-P as measured by the P content of fraction IV (Table I) is likely to give erroneously high values, and, in certain cases, the DNA-P may be underestimated (see addendum, ref. 228 p. 45).

[d] Skeletal muscles taken from the hind legs and shanks.

TABLE III

AMOUNTS OF RIBONUCLEIC ACID PHOSPHORUS (PNA-P) AND DEOXYRIBONUCLEIC ACID PHOSPHORUS (DNA-P) IN VARIOUS ANIMAL AND HUMAN TISSUES

Tissue and Species	Weight of organ, g.	Total per organ		Micrograms per 100 mg. fresh tissue		Micrograms per mg. protein N		Picograms per average cell		PNA-P / DNA-P Ratio	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Liver:											
Mouse				93.0	25.5	32.4	8.9			3.62	(62)
Mouse				92.9	27.9	28.1	8.4			3.34	(36)
Mouse				90.0	28.5					3.16	(63)
Mouse				92.7	23.2					4.00	(64)
Rabbit				79	17					4.6	(65)
Rabbit				30.9	7.3					4.26	(66)
Rabbit				31.9	16.1					1.98	(24)
Rabbit										1.41	(67)
Cat				78	34					2.30	(5)
Guinea pig				97	42					2.30	(53)
Pullets	45.0	37.8	14.0	84	31.1					2.70	(39)
Cockerel	37.8	29.3	15.6	77.5	41.3					1.86	(39)
Monkey				29	8.4					3.45	(68)
Man				52	21	21.4	8.8	2.48	1.0	2.48	(69)
Pancreas:											
Ox				100	6.5					15.4	(1)
Ox				177	22					8.1	(5)
Rabbit				119	52					2.3	(5)
Cat				146	43					4.3	(5)
Submaxillary gland:											
Mouse				88.0	58.3	62.5	41.5	2.01	1.34	1.51	(70)
Mouse	0.069	0.051	0.027	75.1	38.5					1.95	(71)
Kidney:											
Mouse										1.0	(62)
Mouse										1.16	(72)
Rabbit				16.7	12.5					1.34	(66)
Rabbit										0.54	(67)
Guinea pig				36	36					1.0	(53)
Man				20	16	27.2	21	1.10	0.83	1.33	(69)
Spleen:											
Mouse										0.30	(72)
Mouse										0.37	(62)
Rabbit										0.32	(67)
Guinea pig				71	91					0.8	(53)
Rabbit				73	91					0.8	(5)
Man				36	77					0.5	(5)

TABLE III (*Continued*).

Tissue and Species	Weight of organ, g.	Total per organ		Micrograms per 100 mg. fresh tissue		Micrograms per mg. protein N		Picograms per average cell		PNA-P / DNA-P Ratio	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Thymus:											
Mouse	0.033	0.020	0.086	62	262					0.24	(73)
Mouse										0.15	(72)
Calf				90	225					0.4	(5)
Lymphoid tissue:											
Mouse				52	180					0.29	(73)
Mouse										0.28	(72)
Bone marrow:											
Rabbit				18	48					0.38	(51)
Man				4.7	5.75			0.69	0.869	0.81	(74)
Leucocytes:											
Rabbit				22.5	95.1					0.24	(75)
Man								0.25	0.734	0.38	(74)
Brain:											
Rabbit										0.73	(67)
Dog, white matter				5.3	6.3					0.84	(76)
gray matter				11.1	5.3					2.10	(76)
Monkey, white matter				6.4	12.4					0.52	(77)
gray matter				11.5	9.6					1.20	(77)
Guinea pig				24	28					0.8	(53)
Sciatic nerve: Cat				3.9	4.8					0.9	(76)
Muscle:											
Rabbit				24	8					3.0	(65)
Rabbit										0.54	(67)
Guinea pig				24.5	10					2.45	(53)
Heart: Guinea pig				27	24					1.1	(53)
Skin: Mouse				11.9	39.3	7.6	25.0	0.22	0.71	0.30	(70)
Thyroid: pig										0.52	(78)
Crop gland: Pigeon	1.53	0.50	0.38	32.6	25.1					1.30	(79)
Brain:											
Carp						20.6	16.0	0.45	0.35	1.28	(80)
Tortoise						29.6	9.1	1.63	0.50	3.26	
Duck						10.6	6.3	0.44	0.26	1.69	
Fowl						17.0	8.3	0.45	0.22	2.05	
Rat						9.7	8.2	0.72	0.61	1.18	
Guinea pig						18.5	7.9	1.61	0.69	2.33	
Cat						8.9	7.8	0.79	0.69	1.14	
Dog						8.2	7.0	0.79	0.67	1.18	
Man						27.4	7.1	2.63	0.68	3.87	

The ratio PNA-P/DNA-P is much higher for liver and pancreas than for other tissues. Tissues with lower PNA-P/DNA-P ratios which still exceed 1.5 are testis, seminal vesicles, and sub-maxillary gland (Tables II and III). It will be noted that the function common to all the above tissues is their active formation of proteins or nucleoproteins, and, since the DNA is confined to the nucleus and the chromosomes, the high PNA-P content would appear to be related in some way to this type of cell activity. This, of course, is the view that was originally developed in the pioneer investi-

[47] M. Fukuda and A. Shibatani, *J. Biochem. (Japan)* **40,** 95 (1953).
[48] P. Mandel, M. Jacob, and L. Mandel, *Bull. soc. chim. biol.* **32,** 80 (1950).
[49] M. R. Sahasrabudhe and M. V. Lakshminarayan Rao, *Nature* **168,** 605 (1951).
[50] W. C. Schneider and H. L. Klug, *Cancer Research* **6,** 691 (1946).
[51] C. Lutwak-Mann, *Biochem. J.* **49,** 300 (1951).
[52] M. Rabinovitch, L. C. U. Junquiera, and H. A. Rothschild, *Science* **114,** 551 (1951).
[53] J. Rerabek, *Arkiv Kemi, Mineral. Geol.* **24,** 1 (1947).
[54] M. E. Lombardo, L. R. Cerecedo, and D. V. N. Reddy, *J. Biol. Chem.* **202,** 97 (1953).
[55] I. A. Rose and B. S. Schweigert, *Proc. Soc. Exptl. Biol. Med.* **79,** 541 (1952).
[56] P. Mandel, L. Mandel, and M. Jacob, *Compt. rend.* **232,** 1513 (1951).
[57] H. von Euler and L. Hahn, *Arch. Biochem.* **17,** 285 (1948).
[58] E. Hoff-Jørgensen, *Biochem. J.* **50,** 400 (1951).
[59] P. Mandel, M. Jacob, and L. Mandel, *Compt. rend. soc. biol.* **143,** 536 (1949).
[60] M. Jacob, L. Mandel, and P. Mandel, *Experientia* **7,** 269 (1951).
[61] W. A. Rambach, D. R. Moomaw, H. L. Alt, and J. A. D. Cooper, *Proc. Soc. Exptl. Biol. Med.* **79,** 59 (1952).
[62] A. J. Baxi, K. D. Samarth, and P. R. Venkataraman, *Proc. Indian Acad. Sci.* **34,** 258 (1951).
[63] C. P. Barnum, C. W. Nash, E. Jennings, O. Nygaard, and H. Vermund, *Arch. Biochem.* **25,** 376 (1949).
[64] R. M. Johnson and S. Albert, *J. Biol. Chem.* **200,** 335 (1953).
[65] J. M. Young and J. S. Dinning, *J. Biol. Chem.* **193,** 743 (1951).
[66] C. U. Lowe, W. L. Williams, and L. Thomas, *Proc. Soc. Exptl. Biol. Med.* **78,** 818 (1951).
[67] F. Gros, S. Bonfils, and M. Machebeouf, *Compt. rend.* **233,** 990 (1951).
[68] C. U. Lowe and C. P. Barnum, *Arch. Biochem. and Biophys.* **38,** 335 (1952).
[69] J. N. Davidson, I. Leslie, and J. C. White, *Lancet* **i,** 1287 (1951).
[70] W. G. Wiest and C. Heidelberger, *Cancer Research* **13,** 250 (1953).
[71] M. Rabinovitch, H. A. Rothschild, and L. C. U. Junquiera, *J. Biol. Chem.* **194,** 835 (1952).
[72] M. E. Lombardo, J. J. Travers, and L. R. Cerecedo, *J. Biol. Chem.* **195,** 43 (1952).
[73] P. P. Weymouth and H. S. Kaplan, *Cancer Research* **12,** 680 (1952).
[74] J. N. Davidson, I. Leslie, and J. C. White, *J. Pathol. Bacteriol.* **63,** 471 (1951).
[75] N. S. Burt, R. G. E. Murray, and R. J. Rossiter, *Blood* **6,** 906 (1951).
[76] J. E. Logan, W. A. Mannell, and R. J. Rossiter, *Biochem. J.* **51,** 482 (1952).
[77] D. Bodian and D. Dziewiatkowski, *J. Cellular Comp. Physiol.* **35,** 155 (1950).
[78] J. Rerabek, *Biochem. et Biophys. Acta* **8,** 389 (1952).
[79] W. H. McShan, J. S. Davis, S. W. Soukup, and R. K. Meyer, *Endocrinology* **47,** 274 (1950).
[80] R. Bieth and P. Mandel, *Experientia* **9,** 185 (1953).

gations of Brachet[81] and Caspersson,[82] and it is discussed at length in Chapter 28. A recent report that the PNA/DNA ratio does not vary during the secretory cycle of the pancreas[83] apparently complicates the situation.

Because DNA is located in the nucleus and is present in constant amount in each diploid cell, it follows that the concentration of DNA-P per unit of fresh weight will be high in tissues with little cytoplasm and extracellular material and low when the proportion of cytoplasmic substance is relatively large. On this basis, the proportion of chromosomal material in the cells can be seen to decrease according to the following scale: thymus (about 250 μg. DNA-P/100 mg.), lymphoid tissue (180 μg.), rat bone marrow (140 μg.), small intestine (130 μg.), spleen (100 μg.), leucocyte (95 μg.), lung (75 μg.), submaxillary gland (50 μg.), pancreas (45 μg.), liver and kidney (30 μg.), heart, testis, and seminal vesicles (25 μg.), and brain and skeletal muscle (10 μg. or less). The thymolymphatic system forms a distinct group characterized by high concentrations of DNA (above 90 μg.), whereas tissues which are actively functioning in a synthetic, mechanical, or nervous capacity all have low concentrations of DNA-P (50 μg. or less) and relatively large amounts of cytoplasm.

The concentrations of PNA-P in the tissue per unit of fresh weight are distributed in a way which cuts across one of the above divisions. In this case the group with high PNA-P concentrations (i.e., above 50 μg. PNA-P/100 mg. fresh weight) includes liver, pancreas, small intestine, and the cells of the reticulo-endothelial system. Denucé[84] has shown that the concentration of PNA is much higher in the secretory than in the storage portion of the silk glands of *Bombyx mori*. The tissues with the lowest PNA-P concentrations include the group of active "mechanical" organs, namely, kidney, heart, and skeletal muscle. Within this last group Mandel reports that heart tissue from a variety of species always has a higher PNA concentration than the corresponding, and less active, skeletal muscle.[85] Mandel and his colleagues have also reported values for the concentration of nucleic acids in milk, blood, and plasma.[86a,b,c]

Any interpretations on the basis of concentration per unit weight of tissue are complicated by the differences in the distribution and activity of intracellular PNA. In rat liver cells, 85 to 90 % of the PNA is located in the cytoplasm, the remainder being in the nucleus (Table VII, and Chapter 21).

[81] J. Brachet, (a) *Enzymologia*. **10,** 87 (1941); (b) *Arch. biol. (Liége)* **53,** 207 (1941).
[82] T. Caspersson, (a) *Chromosoma* **1,** 562 (1940); *Naturwissenschaften* **29,** 33 (1941).
[83] M. M. Daly and A. E. Mirsky, *J. Gen. Physiol.* **36,** 243 (1952).
[84] J. M. Denucé, *Biochim. et Biophys. Acta* **8,** 111 (1952).
[85] P. Mandel, *Exposés ann. biochim. méd.* **13,** (1951).
[86] (a) P. Mandel and R. Bieth, *Compt. rend. soc. biol.* **142,** 234 (1948); (b) P. Mandel, P. Metais, and R. Bieth, *Compt. rend. soc. biol.* **142,** 1022 (1948); (c) P. Mandel and P. Metais, *Compt. rend. soc. biol.* **142,** 241 (1948).

The ratio PNA-P/DNA-P for liver nuclei is, however, 0.2 to 0.4, and it is possible that a large part of the total PNA of reticulo-endothelial tissues lies within the nucleus, since in these cells the cytoplasm is scanty and the PNA-P/DNA-P ratio is very low.

When the concentration of PNA-P (including nonnucleotide concomitants) per unit weight of protein nitrogen (PN) is considered, distinct differences between organs are no longer apparent. For adult rat liver, kidney, brain, and spleen (Table II), the figures available lie entirely within the range 25 to 37 μg. PNA-P/mg. PN, in contrast to the wide scatter of other measurements on these tissues. Values within the range 20 to 40 μg. PNA-P/mg. PN are also found in mouse and human liver and in human kidney, but exceptions to the rule are mouse submaxillary gland and skin, and brain tissue (Table III). Much more information on this aspect of tissue composition is clearly essential.

The values for the concentration of DNA-P per unit PN are remarkably similar for homologous tissues in different species.In rat, mouse, and human liver the values are close to 8 μg. DNA-P/mg. PN, and for the brain tissue of tortoise, duck, fowl, rat, guinea pig, cat, dog, and man (carp is the exception), the values [calculated from results of Bieth and Mandel[80]] lie within the narrow range of 6.3 to 9.1 μg. DNA-P/μg. PN.This is all the more significant, as the original figures of Bieth and Mandel for the amounts per cell show DNA, PNA, and PN values ranging from 2.2 to 6.8, 4.4 to 26.3, and 26.4 to 88.4 mg., respectively. The relative constancy of DNA per unit PN leads to the conclusion that for one particular type of tissue, the amount of protein per cell is directly proportional to the DNA content of the nucleus, and hence to chromosome substance.

Mirsky and Ris[87] have suggested that a similar relationship exists between the cell mass and the DNA-P content per nucleus in homologous tissues. The values for the concentration of DNAP/100 mg. fresh tissue (the reciprocal of which is an approximation to the cell mass) give some support to this contention, but they are rather more variable than, for example, the concentration of DNA-P per unit PN in liver and kidney (Tables II and III).

Table IV shows how the amounts of PNA and DNA in tissues are affected by the sex of the animal. In *Drosophila* salivary gland[42] and whole larvae[88] and in fowl liver[39] the PNA-P/DNA-P ratio is greater in the cells of the female than of the male; in the salivary gland, the amounts of DNA-P per cell are not greatly different, and there is more PNA-P per cell in the female than in the male tissue. In each animal the total PNA-P per organ or per larva is also larger in the female than in the male. The situation is completely reversed in rat liver, where, with the exception of

[87] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **34,** 451 (1951).
[88] I. Leslie, E. C. R. Reeve, and F. W. Robertson. in press.

TABLE IV

AMOUNTS OF RIBONUCLEIC ACID PHOSPHORUS (PNA-P) AND DEOXYRIBONUCLEIC ACID PHOSPHORUS (DNA-P) IN VARIOUS FORMS OF MALE AND FEMALE ANIMALS

Tissue (species) and sex	Weight of organ, g.	Total per organ		Micrograms per 100 mg. fesh tissue		Micrograms per mg. protein N		Picograms per average cell		Ratio PNA-P / DNA-P	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Salivary gland (*Drosophila*):											(42)
Male		33.8	3.9			66		225	25.7	8.75	
Female		42.6	4.2			64		283	28.1	10.0	
Giant salivary gland (*Drosophila*):											(42)
Male		44.0	4.2			56		292	30.1	9.70	
Female		54.3	4.5			54		361	29.6	12.2	
Whole larva (*Drosophila*):											(88)
Male	1.64	0.69	0.04	42	2.92	54.8	3.81			14.4	
Female	1.95	1.04	0.061	53	3.12	58.2	3.41			17.1	
Liver (fowl):											(39)
Male	37.8	29.3	15.6	77.5	41.3			0.44	0.235	1.85	
Female	45.0	37.8	14.0	84.0	31.3			0.64	0.235	2.70	
Liver (rat):											(14)
Male	7.67	7.19	1.66	93.2	21.6	36.9	8.5	3.93	0.913	4.38	
Female	6.55	6.56	1.80	99.9	27.4	39.2	10.8	3.44	0.942	3.64	
Liver (rat):											(89)
Male				96	21					4.3	
Female				108	26					4.0	
Liver (rat):											(90)
Male		10.65	2.40				8.0			4.44	
Female		9.6	2.31				9.8			4.16	
Liver (rat):											(47)
Male	12.0	9.21	2.02	77.5	16.8	33.5	7.25	5.25	1.14	4.62	
Female	9.3	6.71	1.62	72.1	17.4	31.9	7.7	4.65	1.13	4.13	

the DNA-P per cell, all these values are consistently higher for males than for females (Table V, refs.[14, 47, 89, 90]). In addition to demonstrating these differences in liver cell composition of male and female rats, Campbell and Kosterlitz[90] also found that both the PNA-P per unit PN and the ratio

[89] M. F. Harrison, *Proc. Roy. Soc.* (*London*) **B141**, 203 (1953).
[90] R. M. Campbell and H. W. Kosterlitz, *J. Endocrinol.* **6**, 308 (1950).

PN/DNA-P (i.e., the average protein content of the cells) are significantly greater in males than females.

Whatever the agents directly influencing metabolism in the male and female, the possibility exists that these differences between species are related to differences in the nature of the sex-linked chromosomes, between, say, *Drosophila* (females *2X*, males *X* large Y) on the one hand, and rat (females *XX*, males *X* small *y*) on the other. Although Callan[91] found no change in PNA concentration in females with an extra Y chromosome, it can be seen in Table IV that the concentration of PNA-P per unit of fresh tissue does not show the same consistent trend that is found for the PNA-P/DNA-P ratio in different sexes.

IV. Embryonic Development and Postnatal Growth

Embryonic blood and liver cells of the chick have much higher concentrations of total nucleotide than the corresponding adult cells,[94] and in sheep tissue the total nucleic acid concentration per unit dry weight is always higher for embryonic than for adult tissue.[95] Although these facts have been taken to indicate that nucleic acids are associated with the rapid cellular proliferation and protein synthesis characteristic of embryonic tissues, changing concentrations during growth are to some extent deceptive.[13] In terms of amounts per cell, it can be seen in Table V[13, 92] that in chick embryo liver and muscle the PNA-P content of the cell actually increases or remains unchanged during a period when the concentration per unit fresh weight is diminishing. The fall in nucleic acid concentration in the cytoplasm consequently results from dilution of PNA by protein or other cell constituents.

When measurements are made on such a complex system as the whole embryo, interpretations are even more difficult. It has been suggested that, in the developing chick embryo and mouse embryo, the coincidence of high PNA and DNA concentrations with high concentrations of protein support the view that nucleic acids are involved in protein synthesis.[96, 97] Although higher concentrations of PNA are often found in embryonic tissues, this is not invariable (Table V,[8, 13]), and they may not always accompany the phase of rapid accumulation of protein.[98, 99]

[91] H. G. Callan, *Nature* **161,** 440 (1948).
[92] H. Herrmann, *Ann. N. Y. Acad. Sci.* **55,** 99 (1952).
[93] I. Geschwind and C. H. Li, *J. Biol. Chem.* **180,** 467 (1949).
[94] T. Caspersson and B. Thorell, *Chromosoma* **2,** 132 (1941).
[95] J. N. Davidson and C. Waymouth, *Biochem. J.* **38,** 39 (1944).
[96] A. B. Novikoff and V. R. Potter, *J. Biol. Chem.* **173,** 233 (1948).
[97] D. V. N. Reddy, M. E. Lombardo, and L. R. Cerecedo, *J. Biol. Chem.* **198,** 267 (1952).
[98] J. B. Flexner and L. B. Flexner, *J. Cellular Comp. Physiol.* **38,** 1 (1951).
[99] H. Herrmann and J. S. Nicholas, *J. Exptl. Zool.* **112,** 341 (1949).

TABLE V

AMOUNTS OF RIBONUCLEIC ACID PHOSPHORUS (PNA-P) AND DEOXYRIBONUCLEIC ACID PHOSPHORUS (DNA-P) IN EMBRYONIC TISSUES

Tissue (species) and age	Weight of organ, mg.	Total per organ		Micrograms per 100 mg. fresh tissue		Micrograms per mg protein N		Picograms per average cell		Ratio PNA-P / DNA-P	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Brain (chick embryo)											(13)
8 days	56	28	16	50	29	70	40.5	0.41	0.235	1.72	
13 days	241	96	30	40	12	59	18.3	0.75	0.235	3.2	
Hatching	816	371	94	45	12	45	11.3	0.93	0.235	3.75	
2-day chick	822	434	86	53	10	45	9.0	1.18	0.235	5.30	
Brain (chick embryo)											(8)
10 days	195	56	29	29	15	51	24.5			1.91	
13 days	388	125	45	32	12	47	17.1			2.80	
16 days	596	234	72	39	12	45	14.6			3.26	
19 days	835	343	89	41	11	43	11.1			3.86	
Liver (chick embryo)											(13)
8 days	11	13	3	118	27	110	25.2	1.02	0.235	4.4	
15 days	215	185	51	86	24	60	16.8	0.85	0.235	3.58	
Hatching	872	788	163	90	19	52	11.0	1.14	0.235	4.74	
2-day chick	1278	1133	240	89	19	46	9.9	1.11	0.235	4.68	
Heart (chick embryo)											(13)
8 days	4	2	0.7	50	17	58	19.5	0.67	0.235	2.94	
14.5 days	68	32	12	47	18	72	27.4	0.63	0.235	2.61	
Hatching	237	116	48	49	20	45	18.4	0.57	0.235	2.45	
2-day chick	310	118	59	38	19	29	14.3	0.47	0.235	2.00	
Muscle (chick embryo)											(13)
11.5 days	—	—	—	45	21	110	51	0.49	0.235	2.16	
17.5 days	—	—	—	47	20	49	21	0.56	0.235	2.35	
Hatching	—	—	—	36	12	25	8.2	0.73	0.235	3.00	
2-day chick	—	—	—	38	14	20	7.3	0.64	0.235	2.72	
Muscle (chick embryo)											(92)
9 days	—	—	-	39	19	74	37	0.53	0.26	2.03	
16 days	—	—	-	30	20	35	23.2	0.39	0.26	1.50	
18 days	—	—	—	34	23	29	19.4	0.38	0.26	1.48	
20 days	—	—	—	24	12	21	10	0.55	0.26	2.10	
Fetal liver (rat)	0.026									0.98	(93)
19 days	0.140									0.93	
21 days	0.245									1.94	
Liver (young rat)											
1 day	0.310									1.43	
40 days	4.6									2.83	
Liver (rat)											(47)
10 days	0.30	0.17	0.098	58.4	33.3	35.0	19.9	1.04	0.59	1.76	
21 days	0.98	0.50	0.223	52.2	23.6	26.6	12.0	1.16	0.51	2.29	
Male: 41 days	5.7	4.84	1.17	85.3	20.7	43.0	10.5	4.67	1.11	4.21	
80 days	8.1	6.65	1.44	81.5	17.7	33.8	7.35	5.25	1.14	4.59	
182 days	12.0	9.21	2.02	77.5	16.8	33.5	7.25	5.25	1.14	4.62	
Liver (pullet)											(39)
1 day	0.87	0.49	0.18	56.3	20.7					2.70	
32 days	7.53	4.2	2.31	57.1	31.4					1.82	
123 days	30.0	22.8	9.63	76.0	32.1					2.36	
180 days	45.0	37.8	14.0	84.0	31.1					2.70	
Cerebral cortex (guinea pig)											(98)
25th day of gestation										1.0	
Adult										3.3	

The concentration of PNA-P per unit PN is one feature which shows the same downward trend for all the embryonic tissues (Table V). In these tissues (brain, heart, liver, and muscle) the protein is accumulating more rapidly than the PNA, in contrast to the more even balance that is maintained during the postnatal growth of rat liver. However, when the body weight of the rat is between 20 and 50 g., the rate of PNA accumulation exceeds that of protein, resulting in a peak in the PNA-P/PN ratio[47].

In chick embryo tissue, the relative accumulation rates of various P constituents were obtained by plotting the total amounts against total DNA-P and so obtaining the value k in the heterauxetic equation $y = bx^k$.[13] In liver and brain, the rates for PNA-P and PN slow down to new constant values between the fourteenth and fifteenth days, the accumulation of PNA lagging behind that of protein in each phase. For the first five days of development of the chick embryo, Herrmann[92] reports a close correspondence between the accumulation rates of PNA, DNA, and PN. It is in the later stages, during the differentiation of the embryonic tissues, that the protein is building up more rapidly than the PNA. During the early development of amphibian embryos Brachet[100] reported simultaneously high concentrations of PNA, DNA, and ATP in the dorsal half of the gastrula when this was developing more rapidly than the ventral half.

From decreasing concentrations of DNA-P per unit weight and per unit PN, one can deduce that cell size and protein content are steadily increasing. This is the case in chick embryonic brain, liver, and muscle, but not in heart tissue, which is morphologically fully developed at the eighth day of incubation.[101] In the cerebral cortex of the guinea pig, the DNA concentration per unit weight of cortex is seven times more at the twenty-fifth day of gestation than in the adult,[97] a fact which agrees with the observed changes in number of cell nuclei per unit volume of cortex.[102]

On the assumption that the increase in DNA content of brain tissue is actually measuring increasing cell number, Mandel and Bieth[103] reported that the cell number of rat brain reaches its final adult level 16 days after birth. Guinea pig brain has developed to this stage at the time of birth, cat and rabbit brain at 1 month, dog brain at 5 months, and human brain at approximately 1 year.[104]

In embryonic chick brain and adult rat liver, there is a three-and five-fold increase in the PNA-P per cell over their respective developmental stages (Table V). Such changes are, of course, reflected in the ratio PNA-P/

[100] J. Brachet, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 18 (1947).
[101] F. R. Lillie, "The Development of the Chick." Henry Holt and Co., New York, 1908.
[102] V. B. Peters and L. B. Flexner, *Am. J. Anat.* **86,** 133 (1950).
[103] P. Mandel and R. Bieth, *Experientia* **7,** 343 (1951).
[104] P. Mandel and R. Bieth, *Compt. rend.* **235,** 485 (1952).

DNA-P, as Bieth *et al.*[8] noted in the case of chick embryo brain. Higher values for the PNA-P/DNA-P ratio in the adult as compared with the embryonic tissues have been reported for the cerebral cortex of the guinea pig,[97] skeletal muscle of the chick,[105] and rat liver.[93] The ratio in fetal rat liver,[93] although not in embryonic chick liver,[13] corresponds to those for adult reticulo-endothelial tissues (Tables II and III). The PNA-P/DNA-P ratio is constant in rat kidney, over a period in which cell number increases by 60 %.[106] Brody[107] reports that the ratio is highest during the logarithmic growth phase of the human placenta.

In *Drosophila* larvae, the salivary glands are composed of about 150 cells which can undergo appreciable enlargement without dividing. In their interesting study of this tissue, Patterson and Dackermann[43] report an increase in the average PNA and PN content per cell, but not in DNA content during doubling of cell size (Table IV, ref. 42). A similar situation occurred in the development of spermatocytes in the insect, *Arvelius albopunctatus*,[108] and in the doubling of nuclear volume without increase of DNA content during estrogen stimulation of rat uterus.[109]

The PNA/DNA ratio decreases during the early development of *Arbacia* eggs, because at this stage the total amount of PNA per egg remains unchanged, while the DNA content steadily increases.[32] Using a microbiological method[58] which measures the total deoxyriboside content of amphibian eggs, Hoff-Jørgensen and Zeuthen[110] found that this does not change during the initial segmentation division of the fertilized ovum. These apparently contradictory observations could be reconciled if a conversion of an initial store of free deoxyribosides to polymerized DNA occurred in the first stages of embryonic development.

Although these and other results[111, 112] appear to rule out the possibility of the conversion of PNA to DNA, as originally proposed by Brachet,[113] Agrell has recently reported an inverse relationship between the PNA and DNA contents of the pupae of *Calliphora erythorocephala* during metamorphosis.[114] As the DNA decreases, the PNA increases during histolysis of larval tissues; by the completion of histogenesis, the DNA content has increased again, and the PNA content diminished.

[105] D. S. Robinson, *Biochem. J.* **52,** 628 (1953).
[106] N. B. Kurnick, *J. Exptl. Med.* **94,** 373 (1951).
[107] S. Brody, *Exptl. Cell Research* **3,** 702 (1952).
[108] F. Schrader and C. Leuchtenberger, *Exptl. Cell Research* **1,** 421 (1950).
[109] M. Alfert and H. A. Bern, *Proc. Natl. Acad. Sci.* **37,** 202 (1951).
[110] E. Hoff-Jørgensen and E. Zeuthen, *Nature* **169,** 245 (1952).
[111] C. A. Villee, M. Lowens, M. Gordon, E. Leonard, and A. Rich, *J. Cellular Comp. Physiol.* **33,** 93 (1949).
[112] R. Abrams, *Exptl. Cell Research* **2,** 235 (1951).
[113] J. Brachet, *Compt. rend. soc. biol.* **108,** 813, 1167 (1931).
[114] I. Agrell, *Nature* **170,** 543 (1952).

V. Regenerating Tissues and Tissue Explants

The regeneration of rat liver tissue that follows the removal of the median and left lateral lobes (i.e., roughly two-thirds of the liver) is a truly hyperplastic process,[115, 116] the proliferation of new cells occurring at a rate "which probably exceeds that of liver tumors.[12]" The restoration of liver tissue is so vigorous that the remaining lobe has grown to reach 70 to 80 % of the original liver weight within 4 to 10 days of the operation.[14, 117, 118] Regeneration involves all the cell types found in liver, and after 21 days "the total liver populations of parenchymal cells, sinusoidal lining cells and bile-duct cells have all returned to normal.[117]"

A number of authors, attracted by such an intensive proliferating system, have investigated the changes in nucleic acid composition of regenerating liver. Drabkin[119] noted that the percentage increments of DNA were closely similar to the percentage weight increase during the restoration of rat liver. Since then, the total DNA-P content has been recognized as a measure of cell number, and important progress has been made.[12, 14, 118, 120] As regards the average amount of DNAP-P per nucleus during regeneration, there is general agreement that this increases significantly during the phase of rapid cell proliferation, although there is some doubt about the extent of the increase in the first two days (Table VI). Price and Laird[12] came to the conclusion that the PNA content of the small granular and the supernatant fractions of the liver homogenates also doubled before cell division.

There is a certain lack of agreement so far about the behavior of the PNA-P/DNA-P ratio. All authors, however, agree that the PNA-P content per cell reaches the upper limit during the first four days of regeneration,[12, 14, 120] most of the increase occurring in the small granule fraction. These increases coincide with a period in which liver growth (by weight) is most rapid,[121] in which cell number, as measured by cell counts, is nearly doubled,[116] and total DNA-P content is increased by 70 to 81 %.[54, 118]

The PNA concentration per unit weight reaches its highest level between 1½ and 6 days after partial hepatectomy,[12, 14, 54, 121, 122] the increase occurring in all the intracellular fractions.[12] The significance of this can be judged by the fact that Novikoff and Potter[121] report no corresponding increases in lactic acid, ATP, ADP, or the activity of some of the enzymes associated with the tricarboxylic acid cycle during liver regeneration.

[115] G. M. Higgins and R. M. Anderson, *Arch. Pathol.* **12,** 186 (1931).
[116] A. M. Brues, D. R. Drury, and M. C. Brues, *Arch. Pathol.* **22,** 658 (1936).
[117] M. Abercrombie and R. D. Harkness, *Proc. Roy. Soc. (London)* **B138,** 544 (1951).
[118] G. T. Mills, J. Paul, and E. E. B. Smith, *Biochem. J.* **53,** 245 (1953).
[119] D. L. Drabkin, *J. Biol. Chem.* **171,** 395 (1947).
[120] J. E. Ultmann, E. Hirschberg, and A. Gellhorn, *Cancer Research* **13,** 14 (1953).
[121] A. B. Novikoff and V. R. Potter, *J. Biol. Chem.* **173,** 223 (1948).
[122] P. Drochmans, *Arch. biol. (Liége)* **61,** 475 (1950).

TABLE VI

AMOUNTS OF RIBONUCLEIC ACID PHOSPHORUS (PNA-P) AND DEOXYRIBONUCLEIC ACID PHOSPHORUS (DNA-P) IN RAT LIVER DURING THE COURSE OF REGENERATION AFTER PARTIAL HEPATECTOMY

Experimental conditions	Weight of organ, g.	Total per organ		Micrograms per 100 mg. fresh tissue		Micrograms per mg. protein N		Picograms per average cell		PNA-P / DNA-P Ratio	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Controls						25.3	6.7	3.8	1.0	3.80	(12)
Hepatectomy + 1 day						35.4	10.8	6.1	1.83	3.33	
+ 4 days						41.0	11.4	4.3	1.20	3.58	
+ 8 days						38.2	10.7	4.2	1.18	3.55	
+ 23 days						26.0	6.7	3.9	1.0	3.90	
Controls	7.94	7.35	1.75	91.8	21.9			3.96	0.93	4.27	(14)
Hepatectomy + 1 day	3.02	3.0	0.67	99.5	22.2			4.5	1.02	4.48	
+ 2 days	3.58	3.72	0.77	104	21.5			6.1	1.26	4.83	
+ 4 days	5.18	5.78	1.22	112	23.5			6.0	1.27	4.72	
+ 6 days	5.42	6.24	1.40	115	25.8			4.15	0.94	4.46	
+ 10 days	6.15	6.15	1.45	100	23.6			4.2	0.99	4.25	
Controls								3.06	1.04	2.94	(120)
Hepatectomy + 12 hr.								3.14	1.06	2.96	
+ 24 hr.								4.46	1.40	3.18	
+ 36 hr.								5.11	1.42	3.60	
+ 48 hr.								4.43	1.32	3.35	
+ 60 hr.								3.79	1.26	2.99	

Price and Laird[12] and Thomson *et al.*[14] find that the DNA-P concentration per unit weight rises to a peak value at the fourth to sixth day after the operation. Since the DNA-P per unit PN also reaches a maximal value at the fourth day, cell size and protein content per cell must be minimal at this stage and must gradually increase during the remainder of regeneration. The rise in PNA-P per unit PN (Table VI,[12]) up to the fourth day coincides with rapid cell multiplication, as is also the case in embryonic development (Section IV), and in cultures of *Polytomella caeca* (Section XI).

The increase in the size of the remaining kidney which follows unilateral nephrectomy is usually termed "renal hypertrophy." Mandel and his collaborators find that the increase in kidney size over 80 days is accompanied by a 37 % increase in total DNA-P content and hence, they conclude, a

similar increase in cell number.[123] As "hyperplasia" is usually defined as increase in cell number, and "hypertrophy" as increase in cell size,[124] the more accurate description of the compensatory process would be "kidney hyperplasia."

The situation is made more confusing because Kurnick has briefly reported[125] that growth of the remaining kidney is true "hypertrophy without significant hyperplasia." It seems to be the case that cell size, PNA, and protein content increase between 40 to 50 % at 7 days when there is little increase in DNA content, but that cell composition finally returns to its original state.[123] When the diet is deficient in protein, the cell number or DNA content of the remaining kidney increases to at least the same extent as in the fully fed animals, but the percentage increases in weight, protein, and PNA per kidney are much lower.[126]

Before suitable methods become available for measuring the PNA-P and DNA-P contents of embryonic chick heart explants growing *in vitro*, increases in total NAP during growth were recorded.[127] Once net increases in both PNA-P and DNA-P were produced by a suitable culture technique, it became clear that an increase in PNA-P content always preceded the first rise in the DNA-P content of a group of explants,[128] and that the ratio PNA-P/DNA-P, and hence the PNA content per cell, rose appreciably during intensive cell division.[128-130] Although the average DNA-P content per nucleus certainly increases during rapid cell division,[131] at the end of this phase the values for DNA-P content per individual nucleus show the same distribution in the "fibroblast" cultures as in the 12-day chick embryo liver.[132]

The total increments in nucleic acids and protein in growing cultures may depend on the technique employed. Davidson and Leslie[130] found closely similar percentage increases in PN, PNA-P, and DNA-P contents of the chick heart explants over a period of 10 days. On the other hand, Gerarde *et al.*[133] report that in their lung, heart, and intestine cultures, also prepared from chick embryo, the rise in protein content was three or more times that

[123] P. Mandel, L. Mandel, and M. Jacob, *Compt. rend.* **230,** 786 (1950).
[124] A. E. Needham, "Regeneration and Wound Healing." Methuen, London, 1952.
[125] N. B. Kurnick, *Abstr. 2nd congr. intern. biochim., Paris* p. 363 (1952).
[126] P. Mandel, L. Mandel, and M. Jacob, *Compt. rend. soc. biol.* **144,** 1548 (1950).
[127] J. N. Davidson and C. Waymouth, *Biochem. J.* (a) **37,** 271 (1943); (b) **39,** 188 (1945); (c) **40,** 568 (1946).
[128] J. N. Davidson, I. Leslie, and C. Waymouth, *Biochem. J.* **44,** 5 (1949).
[129] W. Hull and P. L. Kirk, *J. Gen. Physiol.* **33,** 327 (1950).
[130] J. N. Davidson and I. Leslie, *Exptl. Cell Research* **2,** 366 (1951).
[131] P. M. B. Walker and H. B. Yates, *Proc. Roy. Soc. (London)* **B140,** 274 (1952).
[132] S. C. Frazer and J. N. Davidson, *Exptl. Cell Research* **4,** 316 (1953).
[133] H. W. Gerarde, M. Jones, and T. Winnick, *J. Biol. Chem.* **196,** 69 (1952).

TABLE VII

EFFECT OF VARIOUS DIETARY CONDITIONS ON AMOUNTS OF PNA-P AND DNA-P IN DIFFERENT ANIMAL TISSUES

Experimental conditions	Weight of organ, g.	Total per organ		Micrograms per 100 mg. fresh tissue		Micrograms per mg. protein N		Picograms per average cell		PNA-P / DNA-P Ratio	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Liver (rat): Controls				101	21.3	38.5	8.14		1.08	4.75	(134)
Protein-free diet for 1 week				82	26.3	42.5	13.6		1.10	3.12	(135)
Liver (rat): Controls	8.0	7.6	1.8							4.22	(5)
Starved for 2 day	7.1	6.1	1.8							3.39	
Refed for 2 days	8.6	7.2	2.0							3.60	
Liver (rat)											(14)
Stock diet	7.94	7.35	1.75	91.8	21.9	37.2	8.9	3.9	0.913	4.27	
Fasted 72 hr.	4.89	5.33	1.63	109.1	33.4	36.9	11.3	2.9	0.885	3.28	
High-fat diet for 14 days	9.01	6.01	1.82	68.1	20.6	35.4	10.7	2.96	0.889	3.33	
Semisynthetic diet	8.22	7.53	1.73	91.9	21.1	38.6	8.9	3.92	0.893	4.38	
Protein-deficient: 7 day	6.30	5.11	1.60	81.6	25.5	39.2	12.2	3.02	0.943	3.20	
15 days	5.69	5.22	1.67	92.2	29.4	50.0	15.9	2.76	0.872	3.16	
Liver (rat): Controls	5.47	5.58	1.08	102	19.8	36.2	7.0			5.16	(48)
Prolonged protein-deficient diet	2.78	3.11	1.02	112	36.8	46.4	15.2			3.05	
Kidneys (rat): Controls	1.46	0.96	0.396	66	27.0	27.3	11.3			2.46	(48)
Prolonged protein-deficient diet	0.84	0.44	0.361	52	43.0	24.4	20.0			1.21	
Brain (rat): Controls	1.52	0.57	0.206	37	13.5	25.4	9.2			2.74	(48)
Prolonged protein-deficient diet	1.41	0.57	0.197	40	14.0	28.0	9.7			2.86	
Skeletal muscle (rat): Controls	7.41	1.80	0.37	24.5	5.0	38.6	7.9			4.9	
Prolonged protein-deficient diet	3.44	0.59	0.33	17.1	9.5	64.0	35.9			1.8	
Spleen (rat): Controls	0.52	0.40	0.40	77	77	36.4	36.4			1.0	(60)
Prolonged protein-deficient diet	0.17	0.11	0.17	65	100	28.6	44.0			0.65	
Liver (rat): Controls				83.9	26.8	28.2	9.03			3.13	(136a)
Protein-deficient diet				80.3	30.6	33.4	12.8			2.62	
Nuclei: Controls				11.3	25.2					0.42	
Deficient				9.6	32.6					0.31	
Mitochondria: Controls				4.3	—					0.16	
Deficient				5.6	—					0.18	

Microsomes: Controls				41.6	—					1.55	
Deficient				34.9	—					1.14	
Supernatant: Controls				30.4	—					1.13	
Deficient				37.8	—					1.24	
Liver (rat): Controls			1.72		23.1						(137)
Starved			1.66		26.5						
Protein-deficient											
Body wt.: 98–170 g.			1.92		26.9						
380–420 g.			1.80		23.2						
Liver (rat): Controls	8.22	7.53	1.73	91.9	21.1	38.6	8.9	3.92	0.893	4.38	(14)
Thiamine-deficient for 21 days	5.43	5.31	1.83	98.0	33.9	33.3	11.5	2.67	0.912	2.93	
Liver (rat)				Micrograms per 100 g. body wt.							(49)
Vitamin B_{12}-deficient diet, 4 weeks				2.96	0.90	34.8	10.6			3.29	
Fasted 48 hr.											
Refed: − vitamin B_{12}				3.22	0.90	31.9	8.9			3.58	
+ vitamin B_{12}				4.13	1.16	37.9	10.6			3.56	
Liver (rat)				Micrograms per 100 mg. fresh wt.							(55)
Diet + vitamin B_{12}				69.0	29.9					2.31	
− vitamin B_{12}				51.0	23.7					2.15	
Spleen: + vitamin B_{12}				45.3	159					0.28	
− vitamin B_{12}				42.3	163					0.26	
Kidney: + vitamin B_{12}				29.0	32.4					0.89	
− vitamin B_{12}				27.6	31.4					0.87	
Liver (guinea pig): Controls	10.0	6.28	2.66	62.8	26.6	26.9	11.4		0.904	2.36	(138)
Scorbutic animal	8.6	6.24	2.67	72.5	31.1	35.3	15.1		0.911	2.34	
Muscle (rabbit)											(65)
Diet: + vitamin E				24	8					3.0	
− vitamin E				47	18					2.6	
Liver (rabbit)											
Diet: + vitamin E				79	17					4.6	
− vitamin E				95	30					3.2	
Liver (rat)											(15)
Diet with protein, daily energy intake											
820 kg. cal./sq. m.		6.42				33.7					
1570 kg. cal./sq. m.		7.51				35.4					
Diet without protein, daily energy intake											
880 kg. cal./sq. m.		5.26				37.1					
1670 kg. cal./sq. m.		5.48				41.8					

of PNA or DNA over a 14-day growth period. However, they obtained no net increase in DNA over their zero time value.

VI. Dietary Factors Influencing Nucleic Acid Content

The loss of liver tissue, which occurs in rats during a fast or on a protein-deficient diet, involves a decrease in cell volume,[139] the loss of cytoplasmic granules[140] and pentose,[141] but little or no change in the DNA content or cell number. This conclusion has been established by various studies on the effect of dietary deficiency on tissue nucleic acids (Table VIII).

Among the earlier observations, Campbell and Kosterlitz[134] reported a fall in PNA-P concentration per unit weight of rat liver without a corresponding decrease in DNA-P concentration after one week on a protein-free diet; later they reported that they found no change in the DNA-P per liver in rats on high or low fat diets, whether choline was present or not.[142] Previously, Davidson[5] had found that, although liver weight decreased by 11 % and PNA-P content by 20 %, the total DNA-P per liver remained unchanged after 2 days of fasting. According to Thomson *et al.*,[14] the DNA-P content of rat liver and the average amount of DNA-P per nucleus are not significantly altered by a 72-hr. fast, a high fat diet for 35 days, a protein-free diet up to 15 days, or a thiamine-deficient diet for 21 days. By contrast, the average cell mass (liver weight/total DNA-P content) decreases by about 35 %, and the average cell content of phospholipid, PN, and PNA-P by between 20 and 30 % during fasting or protein deficiency.

Mandel and his collaborators extended their earlier observations to the study of the effects of prolonged protein deficiency (50 to 70 days' duration) on the nucleic acid content of various rat tissues (Table VII refs. 48, 60). In most of the tissues (brain, kidney, liver, cardiac, and skeletal muscle) the DNA-P content and (by implication or by actual nuclear counts) the cell number remained unaltered or only slightly increased. Only in the rat spleen was there a considerable reduction (55 %) in the DNA-P content and in the total number of nuclei.[60] The accompanying loss of PNA and

[134] R. M. Campbell and H. W. Kosterlitz, *J. Physiol.* **106,** 12P (1947).
[135] R. M. Campbell and H. W. Kosterlitz, *Science* **115,** 84 (1952).
[136] (a) E. Muntwyler, S. Seifter, and D. M. Harkness, *J. Biol. Chem.* **184,** 181 (1950); (b) S. Seifter, E. Muntwyler, and D. M. Harkness, *Proc. Soc. Exptl. Biol. Med.* **75,** 46 (1950).
[137] G. C. Villela, *Rev. brasil biol.* **12,** 321 (1952).
[138] M. Fukuda and A. Sibatani, *Experientia* **9,** 28 (1953).
[139] H. W. Kosterlitz and I. D. Cramb, *J. Physiol.* **102,** 18P (1943).
[140] H. W. Kosterlitz, *Nature* **154,** 207 (1944).
[141] J. Brachet, R. Jeener, M. Rosseelt, and L. Thonet, *Bull. soc. chim. biol.* **28,** 460 (1946).
[142] R. M. Campbell and H. W. Kosterlitz, *Biochim. et Biophys. Acta* **8,** 664 (1952).

PN content per organ was of the order of 30 to 75 % in liver, kidney, muscle, and spleen. Brain tissue provided a striking contrast, since it lost none of its PNA, DNA, or PN in the course of severe protein deficiency.[48]

Another aspect of liver metabolism in rats has been investigated by Munro and his colleagues.[15] When the diets contained adequate protein, the PNA content per liver increased with the increasing energy intake; for additional carbohydrate, the regression coefficient was +1.96 mg. PNA-P per liver per 1000 kcal., and, for fat, +2.01 mg. PNA-P per 1000 Cal. In the case of rats on a protein-free diet, which, of course, initially reduces the PNA-P content of the liver, the total PNA-P only increased by 5 to 10 % for each 1000 Cal. supplied as carbohydrate; no change was produced by additional fat. At the same time these authors studied the P^{32} uptake by PNA-P and concluded that "protein intake plays a dominant role in determining the amount of PNA per liver, whereas energy intake determines the amount of phosphorus incorporation."

The amount of PNA-P relative to the total cell substances (that is, the concentration per unit fresh weight) decreases on a protein-deficient diet lasting one week, but increases again in liver, at least, during a more prolonged period of deficiency (Table VII,[14, 48, 134]). Drabkin[119] reported a twofold increase in PNA concentration in regenerating rat liver when the animals were fed on a protein-free diet, the control group receiving a full diet. According to Vendrely and Vendrely[143] and Muntwyler *et al.*[136a,b] the loss of PNA (and of protein also) occurs chiefly in the microsomal fraction, which normally contains nearly 50 % of the total PNA of the liver cell. Lagerstedt's work[144] supports the view that PNA loss involves the disappearance of intracellular cytoplasmic particles, although he holds the opinion that the microsomes may be artificial breakdown products of the mitochondria.

Both PNA and DNA concentrations[49, 55] and the number of nuclei per unit weight of liver[55] are significantly reduced when the animals are made deficient in vitamin B_{12}. Since Rose and Schweigert[55] report that the average DNA and PNA contents per cell are unchanged during vitamin B_{12} deficiency, the lower concentrations of both nucleic acids per unit weight *and per unit PN*[49] must be the result of the build-up of protein and perhaps other constituents in the cells, without a corresponding increase in the nucleic acids. Vitamin B_{12} deficiency appears to have no effect on the nucleic acids of kidney or spleen,[55] but addition of vitamin B_{12} to deficient animals increases the basophilia due to PNA in rat nerve cells.[145] In guinea pigs

[143] C. Vendrely and R. Vendrely, *Compt. rend.* **230,** 333 (1950).
[144] S. Lagerstedt, *Acta. Anat.* Suppl. IX (1949).
[145] W. F. Alexander and B. S. Backler, *Proc. Soc. Exptl. Biol. Med.* **78,** 181 (1951).

TABLE VIII

AMOUNTS OF RIBONUCLEIC ACID PHOSPHORUS (PNA-P) AND DEOXYRIBONUCLEIC ACID PHOSPHORUS (DNA-P) IN VARIOUS NEOPLASTIC TISSUES

Tissues	Weight of organ, g.	Total per organ		Micrograms per 100 mg. fresh tissue		Micrograms per mg. protein N		Picograms per average cell		Ratio PNA-P / DNA-P	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Rat:											
Jensen sarcoma				70.5	43					1.6	(53)
Hepatoma				72.3	54.6					1.32	(146)
Flexner-Jobling carcinoma	4.14	2.3	1.5	55	45					1.2	(147, 148)
Rat:											
Hepatoma				54.1	66.7					0.81	(50)
Fexner-Jobling carcinoma				49.6	56.9					0.87	
Jensen sarcoma				53.2	66.3					0.80	
Walker 256 carcino-sarcoma				59.5	66.3					0.89	
Rat: Hepatoma								1.3	1.0	1.3	(12)
Mouse:											(36)
Liver, whole cell				92.9	27.9	28.1	8.4			3.33	
nuclei				10.2	22.4	19.2	42.3			0.45	
Hepatoma, 98/15											(36)
whole cell				97.5	31.6	42.4	13.7			3.09	
nuclei				16.0	25.3	32.2	50.8			0.63	
Man:											
Bone marrow								0.69	0.87	0.81	(74)
Leukemia								0.75	0.86	0.90	
Blood								0.24	0.70	0.35	
Leukemic blood (leucocytes)								0.38	0.70	0.56	

dying from vitamin C deficiency the concentrations in the liver of both nucleic acids (per unit weight and per unit PN) increased appreciably, while the average DNA-P content per nucleus remained unchanged.[138]

One aspect common to all the above deficiencies, except those of vitamin

[146] J. M. Price, E. C. Miller, J. A. Miller, and G. M. Weber, *Cancer Research* **10,** 18 (1950).

[147] G. A. LePage, V. R. Potter, H. Busch, C. Heidelberger, and R. B. Hurlbert, *Cancer Research* **12,** 153 (1952).

[148] E. B. Schoenbach, N. Weissman, B. Goldberg, and J. Fisher, *Proc. Soc. Exptl. Biol. Med.* **80,** 559 (1952).

B_{12} and vitamin C, is the considerable reduction in the PNA/DNA ratio characteristic for the tissue (Table VII). The increase in DNA-P concentration per unit PN in all cases of dietary deficiency, except that of vitamin B_{12}, is an expression of the loss of protein from the cells without a loss of DNA-P. According to Campbell and Kosterlitz[90] the ratio of PN/DNA-P varies linearly with N intake within certain limits of total food intake.

The PNA-P per unit PN is rather more variable in its behavior. It would appear that the synthesis or replacement of PNA and protein can be uncoupled in dietary deficiencies, the loss of protein taking precedence in protein[14, 48, 134] and vitamin C deficiency,[138] and the failure to replace PNA predominating in cases of high fat diet,[14] vitamin B_{12},[49] and thiamine deficiency.[14] The generalization suggested by Fukuda and Sibatani,[47] that "in liver rapid accumulation or loss of protein lags behind the corresponding rise or fall in PNA," would from the evidence reviewed above apply only to variations in the intake of carbohydrate, fat,[15] and the vitamins B_{12} and thiamine. When the diet is deficient in protein or vitamin C, the protein loss from the liver exceeds that of PNA.

VII. Neoplastic Tissues and Chemical Carcinogenesis

In certain respects the composition of neoplastic tissue is much more uniform than that of normal tissues. Greenstein[149] stresses the strong resemblance between the chemical pattern of different tumors, and Potter *et al.*[150] have developed the concept that "normal cells may be converted to cancer cells by the deletion of enzymes that are not essential for the life of the cell." The similarity in the content of nucleic acids in tumor tissues of different origin was noted by Schneider,[151] and this conclusion is supported by the results collected in Tables VIII and IX. For nearly all the tumor cells, and for liver tissue in a precancerous state after administration of chemical carcinogens, the PNA-P/DNA-P ratio is greatly reduced and, in many cases, is in the region of 1 or less.

Cytochemical studies involving the direct observation of the cells in fixed sections often show increased concentrations of PNA in hepatoma cytoplasm in comparison with cells of adjacent healthy tissue.[152, 153] Stowell[154] reported more DNA per unit volume and per cell in most of the tumors he studied. Although quantitative chemical determinations have borne

[149] J. P. Greenstein, "Biochemistry of Cancer," p. 367. Academic Press Inc., New York, 1947.

[150] V. R. Potter, J. M. Price, E. C. Miller, and J. A. Miller, *Cancer Research* **10**, 28 (1950).

[151] W. C. Schneider, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 169 (1947).

[152] T. Caspersson, "Cell Growth and Cell Function." Norton and Co., New York, 1950.

[153] R. E. Stowell, *Cancer* **2**, 121 (1949).

[154] R. E. Stowell, *Cancer Research* **6**, 426 (1946).

TABLE IX

EFFECTS OF DIETARY CARCINOGENS ON THE AMOUNTS OF PNA-P AND DNA-P IN RAT LIVER TISSUE

Experimental conditions	Weight of organ, g.	Total per organ		Micrograms per 100 mg. fresh tissue		Micrograms per mg. protein N		Picograms per average cell		Ratio PNA-P / DNA-P	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Liver: Basal diet	10.7		2.84		26.6		10.5				(155)
3′-Me-DAB + 2 wk.	7.8		3.09		39.6		16.0				
3′-Me-DAB + 8 wk.	10.9		5.51		50.6		20.5				
Liver: Controls, DAB-free diet				83.3	25.5	35.0	10.7			3.3	(156)
With diffuse tumor				71.1	37.1	34.8	15.9			1.9	
Hepatoma				73.3	37.8	35.9	18.5			1.9	
Liver: basal diet				52.4	19.4					2.7	(157)
+ 2-Me-DAB				49.5	17.0					2.9	
+ 3′-Me-DAB				53.5	56.8					0.94	
Liver: Controls	9.0		2.46		27.3		10.8				(158)
2-Acetylaminofluorene											
+ 2 weeks	9.1		2.38		26.1		10.1				
+ 14 weeks	11.0		2.68		24.4		10.3				
+ 24 weeks	20.0		4.36		21.8		9.9				
Tumor	—		—		31.6		16.1				
Liver: Controls				52.2	24.9	25.0	11.9	2.1	1.0	2.1	(159)
DAB-produced hepatoma cells				72.3	54.6	36.3	26.9	1.3	1.0	1.3	
Liver nuclei				8.2	23.8	19.4	57.0	0.34	1.0	0.34	
Hepatoma nuclei				16.7	47.3	25.6	72.0	0.35	1.0	0.35	
Liver, large granules				15.4	—	26.2	—	0.62	1.0	—	
Hepatoma, large granules				7.4	—	34.4	—	0.14	1.0	—	
Liver, small granules				12.0	—	55.5	—	0.48	1.0	—	
Hepatoma, small granules				12.9	—	57.5	—	0.24	1.0	—	
Liver, supernatant				14.6	—	17.5	—	0.58	1.0	—	
Hepatoma, supernatant				32.3	—	35.0	—	0.59	1.0	—	

Liver: Controls				55.1	18.6			2.96	1.0[a]	2.96	(160)
Diet + DAB				39.2	21.9			1.79	1.0	1.79	
Diet + 3′-Me-DAB				50.6	45.0			1.12	1.0	1.12	
Liver, large granule											(160)
Controls				17.7				0.95	1.0[a]		
+ DAB				11.8				0.54	1.0		
+ 3′-Me-DAB				7.5				0.17	1.0		
Liver, small granules											(160)
Controls				17.8				0.96	1.0		
+ DAB				10.0				0.46	1.0		
+ 3′-Me-DAB				10.2				0.23	1.0		
Liver, nuclei											(160)
Controls				5.4	18.7			0.29	1.0[a]	0.29	
+ DAB				3.6	22.0			0.16	1.0	0.16	
+ 3′-Me-DAB				13.6	39.4			0.30	1.0	0.35	

[a] As various authors have shown that the average amount of DNA-P per nucleus is the same in rat hepatoma as in normal rat liver, the amounts per cell in this table have been calculated on the basis of a value of 1.0 pg. DNA-P per cell (see ref. 10).

this out for the concentration of DNA, they reveal that the actual PNA concentration per unit weight does not change appreciably or consistently in most tumors (Tables VIII and IX).

All tumors, however, are characterized by a decrease in the amount of PNA per cell or in the amount of PNA relative to DNA. There is also a consistent increase in the concentration of DNA-P per unit PN, indicating that tumor or precancerous cells contain relatively less protein than the corresponding healthy cells (Tables VIII and IX).

Different chemical and histological patterns are observed in liver tissue during the induction of hepatoma by (a) carcinogenic azobenzene derivatives and (b) N-acetyl-2-aminofluorene (AAF). The relative carcinogenic potencies of some of the azobenzene derivatives are as follows: 4-aminoazobenzene (4-AB) and 2′-methyl-4-dimethylaminoazobenzene (2′-Me-4-DAB) are noncarcinogenic; on the other hand, the relative carcinogenic activity of 4-DAB is 6, and that of 3′-methyl-4-DAB (3′-Me-4-DAB) is 10 to 12.[160]

For the first 8 weeks on a diet containing 3′-Me-4-DAB, there is no increase in liver size, but a large increase in the concentration of DNA and in the number of nuclei per unit volume of tissue.[155] According to Striebich *et al.*[161] the same carcinogen increases the number of nuclei per gram of liver two and a half times but decreases the mitochondria per gram by 40% in the preneoplastic liver. At the same time, the number of nuclei per liver is doubled, and the number of mitochondria per cell reduced by one-fifth.

The feeding of 4-DAB to rats over 5 to 6 months produced little or no change in the total amounts and concentrations of liver protein, phospholipid, and PNA-P, but their ratios to DNA-P were all greatly decreased.[14] In actual hepatomas, these ratios are half as great as in the control livers. Although this change in composition corresponds to the picture of decreasing number of mitochondria described above, Schneider *et al.*[157] claim that this is true only of preneoplastic cells. In tumor cells, they find the same number of mitochondria per cell as in normal cells, with the difference that these mitochondria have "greatly altered biochemical properties."

Laird[162] has described the dramatic change in liver cell population which occurred between the twenty-sixth and twenty-eighth days of feeding rats

[155] A. C. Griffin, W. N. Nye, L. Noda, and J. M. Luck, *J. Biol. Chem.* **176,** 1225 (1948).
[156] G. T. Mills and E. E. B. Smith, *Science* **114,** 690 (1951).
[157] W. C. Schneider, G. H. Hogeboom, E. Shelton, and M. J. Striebich, *Cancer Research* **13,** 286 (1953).
[158] A. C. Griffin, H. Cook, and L. Cunningham, *Arch. Biochem.* **24,** 190 (1949).
[159] J. M. Price, J. A. Miller, E. C. Miller, and G. M. Weber, *Cancer Research* **9,** 96 (1949).
[160] J. M. Price, E. C. Miller, J. A. Miller, and G. M. Weber, *Cancer Research* **9,** 398 (1949).
[161] M. J. Striebich, E. Shelton, and W. C. Schneider, *Cancer Research* **13,** 279 (1953).
[162] A. K. Laird, *Proc. Soc. Exptl. Biol. Med.* **77,** 434 (1951).

with 3′-Me-4-DAB. For the first 26 days the number of nuclei per liver remained about 2000 × 10^6. By the twenty-eighth day the number increased nearly 50 %. The burst in proliferation involved a fall in the average cell mass to nearly half that of the control liver cells; there was a corresponding reduction in PNA and protein of the large granule fraction.

Griffin *et al.*[153] find a relatively small change in concentration of DNA-P per unit weight during AAF carcinogenesis as compared with its large increase in the precancerous tissue produced by 3′-Me-4-DAB. However, there is a brief report that AAF fed to mice increased the liver DNA concentration by 22 % in 6 months, and by 236 % after 12 months.[163]

Although the concentrations of PNA and protein in the whole tissue are much the same for normal liver and hepatoma, the similarity conceals an actual decrease in amount per cell and a redistribution within the intracellular fractions. As the DNA per nucleus is unchanged in hepatoma cells (see Chapter 19), it can be seen that the entire loss of PNA occurs in the large and small granules, the absolute content per cell of the nuclear and supernatant PNA remaining the same (Table IX[159]).

The most potent carcinogen (3′-Me-4-DAB) causes the greatest reductions in the PNA content of the large and small granules, and the greatest increase in the DNA concentration per unit fresh weight (Table IX[160]). On feeding 2′-Me-4-DAB, which is noncarcinogenic, Striebach *et al.*[161] report a threefold increase in the number of mitochondria per cell and slightly lower concentrations of PNA and DNA in the tissue.

The PNA content of the nucleus is greatly increased in spleen cells of mice carrying transplanted leukemia, but not to the same extent in mice with spontaneous leukemia.[164] In Ehrlich ascites tumor and in lymphoma cells, whatever the amount of DNA per nucleus, the PNA/DNA ratio is always higher in tumor nuclei than in nuclei from healthy cells.[165]

Although fasting produces a decrease in the PNA/DNA ratio in the Flexner-Jobling carcinoma of rats, the tumor continues to grow at the expense of the tissues of the host.[147] However, the rise in PNA/DNA in rat liver tissue after administration intraperitoneally of 1,2,5,6-dibenzanthracene, is not altered by restriction of dietary protein.[166] The virulence of mouse ascites tumor cells appears to decline as PNA is lost from the cells during storage at 4°.[167]

Rerabek[53] and von Euler and Hahn[57] reported somewhat higher PNA/DNA ratios in the tissues of rats bearing Jensen sarcoma. The presence of Crocker sarcoma 180 in mice increased the DNA concentration in liver by

[163] M. E. Lombardo and L. R. Cerecedo, *Federation Proc.* **12,** 241 (1953).
[164] M. L. Peterman and R. M. Schneider, *Cancer Research* **11,** 485 (1951).
[165] C. Leuchtenberger, G. Klein, and E. Klein, *Cancer Research* **12,** 480 (1952).
[166] L. A. Elson, *Symposia Soc. Exptl. Biol.* **3,** 327 (1949).
[167] E. Klein, N. B. Kurnick, and G. Klein, *Exptl. Cell Research* **1,** 127 (1950).

81 % and the PNA by 28 %, thus decreasing the PNA/DNA ratio.[72] Treatment of mice carrying the transplantable sarcoma 180 with aminopterin reduced the PNA and DNA concentrations and increased the PNA/DNA ratio in the tumor tissue.[148]

VIII. Hormonal Influences

The changes in nucleic acid content after administration of various hormones are summarized in Table X, and those following the surgical removal of glands are summarized in Table XI.

After the daily injection of 20 mg. cortisone acetate for 7 days, rabbits were found to have hypertrophied livers, in which the amounts of protein and PNA relative to DNA were greatly increased (Table X[67]). Similar results were reported independently by Lowe *et al.* (Table X[66]). The decrease found in PNA concentration per unit weight indicates that other cell constitutents (e.g., lipids) are building up to a greater extent than PNA. In such circumstances, the basophilia of the cytoplasm will decrease, but it does not follow that PNA synthesis is reduced by cortisone, as the authors suggest.[66]

That fat accumulation is largely responsible for the lower PNA and DNA concentrations is shown by the changes in composition of mouse liver during cortisone treatment.[168] Other experiments of Roberts *et al.*[168] on mice with regenerating livers suggested that "DNA fails to regenerate quickly in livers of cortisone-treated mice."

Inhibition of growth was also obtained on injection of 1.25 mg. cortisone acetate into the chorioallantoic membrane of the 8-day chick embryo.[169] However, in this case, the cortisone lowered the PNA/DNA ratio and decreased the protein content of the cells. Leslie[174] added cortisone to the growth-promoting medium of cultures of embryonic chick heart explants and found slight increases in the total DNA-P synthesis and in the PNA/DNA ratio. When cortisone and growth hormone were combined, the total synthesis of PNA and DNA rose as much as 75 % above normal over 6 days. Growth hormone applied alone slightly inhibited cell multiplication.

According to Gerarde and Jones[175] the nucleic acid *concentrations* did not

[168] K. B. Roberts, H.W. Florey, and W. K. Joklik, *Quart. J. Exptl. Physiol.* **37,** 239 (1952).
[169] C. Cavallero, A. Di Marco, L. Fuoco, and G. Sala, *Proc. Soc. Exptl. Biol. Med.* **81,** 619 (1952).
[170] W. E. J. Phillips, R. H. Common, and W. A. Maw, *Can. J. Zool.* **30,** 201 (1952).
[171] R. M. Campbell, I. R. Innes, and H. W. Kosterlitz, *J. Endocrinol.* **9,** 52 (1953).
[172] M. A. Telfer, *Arch. Biochem. and Biophys.* **44,** 111 (1953).
[173] I. Leslie and J. N. Davidson, *Biochem. J.* **49,** Proc. xli (1951).
[174] I. Leslie, *Biochem. J.* **52,** Proc. xxi (1952).
[175] H. W. Gerarde and M. Jones, *J. Biol. Chem.* **201,** 553 (1953).

change in their cultures of chick embryo lung and intestine after the addition of cortisone acetate to the medium.

Mandel *et al.*[56] report that, after the injection of thyroxine (1 mg. per days) into rats, kidney weight increased 21 to 32 %, protein content 15 to 26 %, PNA 26 to 33 %, and DNA 22 to 32 %. Similar increases in spleen ranged from 35 to 60 %.[176] The absence of any change in cell composition indicates that increase in kidney size was solely the result of cell proliferation (hyperplasia). A single injection of thyroxine into male mice has the effect of raising the proportion of PNA and protein relative to DNA in the liver after 48 hr.[62] As the PNA-P per unit PN continues to increase until 120 hr., total PNA formation appears to exceed the formation of new protein at this stage.

The treatment of immature pullets[177] with estrogen increases liver weight and total liver protein. The treatment was shown to raise significantly the number of hepatic cells and the total DNA per liver,[39] although not to the extent that it increased PNA content. Campbell *et al.*[171] reported similar results for rat liver after injection of estradiol into the intact, but not into the hypophysectomized, animal (Table X, ref. 171). Forty-eight hours after a single injection of estradiol into rats, there is a 192 % increase in PNA per uterus, but only a 12 % increase in DNA content; at 72 hr. the PNA-P increase over the initial amount per uterus is reduced to 137 %, while the corresponding DNA-P increase has risen to 54 %.[172] Testosterone had no effect on the pullet liver; neither did testosterone nor progesterone influence the action of estradiol.[170] Both Mandel *et al.*[178] and Common *et al.*[39] find higher nucleic acid concentrations in the blood serum during estrogen treatment.

The stimulation of pigeon crop gland by lactogenic hormone has been studied by McShan *et al.*[79] From their data it can be shown that after 5 days of treatment the total weight of the gland increased by 280 %, the PNA-P content by 850 %, and DNA-P by 400 %. These authors noted that DNA-P content per organ increased on the second and third days, when the histological picture showed maximal mitotic activity.

Insulin, acting at concentrations of 2 to 3 units/ml. in the growth-promoting medium, increased the total DNA-P and PN contents of chick heart explants well above the control values after 6 days' growth; the stimulus to PNA synthesis was even greater, the PNA-P/DNA-P ratio and the PNA-P per unit PN both increasing appreciably (Table X[173]). Thus, cortisone,[66, 67] insulin,[173] estradiol,[170] and thyroxine[62] all produce a rise in

[176] L. Mandel, M. Jacob, and P. Mandel, *Experientia* **8,** 426 (1952).

[177] D. G. Chapman, A. A. Hanson, R. H. Common, and W. A. Maw, *Can. J. Research.* **27,** 200 (1949).

[178] P. Mandel, J. Clavert, and R. Bieth, *Compt. rend. soc. biol.* **141,** 1262 (1947).

TABLE X

EFFECTS OF HORMONES ON THE AMOUNTS OF PNA-P AND DNA-P IN ANIMAL TISSUES

Experimental conditions	Weight of organ, g.	Total per organ		Micrograms per 100 mg. fresh tissue		Micrograms per mg. protein N		Picograms per average cell		Ratio PNA-P / DNA-P	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Liver (rabbit)						19.8[a]	14[a]			1.41	(67)
2 mg. cortisone for 7 days						20.8	9			2.3	
Liver (rabbit)				30.9	7.3	9.0	2.2			4.26	(66)
Cortisone + 2 days				19.9	3.2	9.6	1.5			6.19	
+ 6 days				22.8	2.8	12.2	1.5			8.19	
Kidney (rabbit)				16.7	12.5	—	—			1.34	
Cortisone + 3 days				17.2	11.3	—	—			1.51	
Liver (mouse): Controls				123	40					3.08	(168)
Cortisone, 2.25 mg., 6 days				106	25					4.24	
Embryo (chick): Controls, 11–18 days				23.4	13.9	19.7	11.7			1.68	(169)
Cortisone, 0.25 μg. on 8th day				18.5	12.9	19.5	13.6			1.43	
Liver (mouse)											(62)
Thyroxine (4 mg./kg.)				93	25.5	32.4	8.9			3.62	
+ 48 hr.				118.5	21.2	34.3	6.1			5.59	
+ 120 hr.				122.7	24.7	41.3	8.3			4.97	
+ 240 hr.				100.4	25.1	33.9	8.5			4.00	
Kidney (rat)	0.694	0.484	0.213	69.7	30.7	34.3	15.1			2.27	(56)
Thyroxine, 1 mg., for 15 days	0.915	0.610	0.277	66.5	30.2	34.3	15.6			2.20	
Liver (pullet)	19.0	13.5	5.75	71.0	30.0	24.4	10.4	0.57	0.235	2.44	(170)
Testosterone (12 × 1 mg.)	18.8	14.3	6.54	75.7	34.4	26.3	12.1	0.53	0.235	2.25	
Estradiol (12 × 1 mg.)	31.0	26.1	7.70	86.0	24.5	29.4	8.7	0.84	0.235	3.56	
Estradiol + progesterone	31.0	25.8	7.35	79.6	22.8	27.2	7.8	0.83	0.235	3.54	

Liver (rat): Controls		8.93	2.17				9.7			4.12	(171)
Estradiol 10 I.U. twice daily for 14 days		9.53	2.33				10.1			4.09	
Estradiol 100 I.U. twice daily for 14 days		12.2	2.47				9.1			4.93	
Progesterone, 2 mg. twice daily for 14 days		11.5	2.63				9.5			4.37	
Hypophysectomized 1 wk., Estradiol at 100 I.U.		5.1	1 82				13.0			2.79	
Uterus (castrated rat)		0.397	1.35							0.29	(172)
Single injection of estradiol											
+ 21 hr.		0.775	1.71							0.45	
+ 48 hr.		1.16	1.51							0.77	
+ 72 hr.		0.94	2.08							0.45	
Crop gland (pigeon)	1.53	0.50	0.38	32.6	25.1					1.30	(79)
Prolactin + 1 day	2.01	0.69	0.46	34.6	22.9					1.51	
+ 5 days	5.82	4.75	1.93	81.7	33.1					2.47	
Heart (chick embryo): Controls		5.48	1.88			29.4	10.1			2.92	(173)
Insulin, 1 unit/ml.		10.38	2.78			39.8	10.6			3.79	
Heart (chick embryo), 48 *in vitro* explants after 6 days growth											(174)
Controls		5.7	1.83							3.1	
Cortisone, 15 μg. ml.		7.5	2.10							3.6	
Growth hormone, 100 μg. ml.		6.3	1.96							3.2	
Cortisone + growth hormone		10.2	3.39							3.0	
Insulin + cortisone + growth hormone		12.6	3.21							3.9	

[a] Per mg. total N of tissue homogenate

TABLE XI

EFFECTS OF REMOVAL OF GLANDS AND PREGNANCY TISSUES ON THE AMOUNTS OF PNA-P AND DNA-P IN VARIOUS ANIMAL TISSUES

Experimental conditions	Weight of organ, g.	Total per organ		Micrograms per 100 mg. fresh tissue		Micrograms per mg. protein N		Picograms per average cell		Ratio PNA-P / DNA-P	Refs.
		PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P	PNA-P	DNA-P		
Liver (rat)				111	25					4.45	(179)
Hypophysectomized				98	26					3.77	
Regenerating liver (normal)				114	27					4.20	
Hypophysectomized				115	38					3.02	
Liver (rat)	5.9	4.85	2.47	82.3	41.9					1.96	(180)
Hypophysectomized	3.84	2.70	1.79	70.2	46.6					1.50	
Liver (rat)				109.3	22.1					4.95	(181)
Hypophysectomized				109.1	27.9					3.91	
Hypophysectomized + growth hormone				116.4	24.2					4.80	
Liver (rat)				102.9	20.2					5.1	(182)
Alloxan-treated				108.9	27.4					3.97	
Liver (rat)	7.60	6.54	1.76	86.2	23.2			3.26	0.874	3.73	(14)
Alloxan-diabetic	6.24	5.79	1.60	93.5	25.7			3.22	0.891	3.62	
Liver (rat)											(11)
Predicted values		10.65	2.40				8.0			4.44	
Castrated males		10.70	2.36				8.55			4.54	
Predicted values		11.4	2.42				7.9			4.72	
Castrated males + testosterone, 2 mg. daily for 17 17 days		11.9	2.39				7.9			4.98	
Predicted values		9.6	2.31				9.8			4.16	
Castrated females		9.2	2.16				9.0			4.27	
Predicted values		10.5	2.42				9.9			4.33	
Castrated females + testosterone		10.0	2.22				8.55			4.52	

Liver (rat); all livers sampled on 21st day										(171)
Nonpregnant females		9.35	2.25				9.7		4.16	
Adrenalectomized, 7 days		9.3	2.34				11.8		3.97	
Ovariectomized, 7 days		8.68	2.15				10.5		4.04	
Hypophysectomized, 2 weeks		8.4	2.25				9.6		3.73	
Normal pregnancy at 21st day		16.4	2.55				8.25		6.44	
Fetuses, placentas, and ovaries removed at 14th day		10.9	2.62				10.5		4.17	
Adrenalectomized at 15th day		16.6	2.79				9.1		5.97	
Hypophysectomized at 15th day		10.7	2.43				11.8		4.39	
Uterus (mouse)		μg.	μg.							(185)a
Proestrus	0.041	27.7	28.1	68	69				0.99	
Estrus	0.080	45.1	30.5	57	40				1.58	
Metestrus	0.075	44.7	34.7	61	49				1.26	
Diestrus	0.032	22.0	23.9	66	70				0.95	
Castrated	0.018	10.4	24.2	56	131				0.43	
Estradiol + progesterone	0.063	26.2	22.2	42	35				1.20	
Seminal vesicles (rat)										(52)
Controls		0.107	0.056	48.5	25.4				1.94	
Castrated		0.024	0.045	25.4	46.9				0.55	
Castrated + testosterone		0.293	0.111	77.2	29.3				2.67	

the proportion of PNA-P to protein in the cells of rat and rabbit liver or embryonic chick heart. When insulin, cortisone, and growth hormone are all present in the medium, the action of insulin in increasing the PNA/DNA ratio is added to the general growth stimulus of the last two hormones.[174]

The control of the pituitary over the nucleic acid content of rat liver has been shown by the fall in PNA-P concentration and in PNA-P/DNA-P ratio following hypophysectomy (Table XI[171, 179, 180, 181]). Di Stefano *et al.*,[181] who found no change in the average DNA per nucleus in the livers of hypophysectomized rats, restored the protein and PNA contents per cell to normal or above normal by injection of growth hormone.

Rats made diabetic with alloxan show a reduction in the liver PNA-P/DNA-P ratio,[14, 182] although in one case the decrease is not significant.[14] There is agreement, however, that the concentrations of liver PNA-P and DNA-P increase in alloxan-treated rats. That protein is lost from the cells follows from the 33 % increase in DNA-P per unit PN[182] and from the fact that the average amount of DNA-P per nucleus remains unchanged.[14]

Since the earlier observations[183] that there is a significant increase in DNA content of rat liver during pregnancy and a remarkable rise in the PNA/DNA ratio, Campbell *et al.* (Table XI[171]) have studied extensively the hormonal effects on rat liver composition. The "excess PNA" (i.e., increase in PNA/DNA above expected value, calculated on the basis of protein content of the cells) is also a feature of liver composition in pregnant mice and guinea pigs but not in cats.[184] "Excess PNA" could be eliminated by removal of fetuses, placentae, and ovaries at the fourteenth day of gestation, or by hypophysectomy, even when food intake was maintained at the normal level.[171] Adrenalectomy caused both the PNA and DNA per liver to increase in pregnant rats. The authors conclude[171] that there are two independent PNA fractions in pregnant rat livers; one varies linearly with protein content, as in nonpregnant rats; the other, "excess PNA," is quite independent of the protein content, or the amount of protein eaten, but "it varies linearly with the amount of energy consumed with the food and with the weight of the placentae."

The PNA/DNA ratio of mouse uterus, which reaches a peak value at estrus, is greatly diminished by castration (Table XI[155a]). It can be restored

[179] A. Canzanelli, R. Guild, and D. Rapport, *Endocrinology* **45**, 91 (1947).
[180] I. Geschwind, C. H. Li, and H. N. Evans, *Arch. Biochem.* **28**, 73 (1950).
[181] H. S. Di Stefano, A. D. Bass, H. F. Diermeier, and J. Tepperman, *Endocrinology* **51**, 386 (1952).
[182] H. F. Diermeier, H. S. Di Stefano, J. Tepperman, and A. D. Bass, *Proc. Soc. Exptl. Biol. Med.* **77**, 769 (1951).
[183] H. W. Kosterlitz and R. M. Campbell, *Nature* **160**, 675 (1947).
[184] R. M. Campbell and H. W. Kosterlitz, *J. Endocrinol.* **9**, 45 (1953).
[185] M. L. Dasher (a) *J. Exptl. Zool.* **119**, 333 (1952) (b) **122**, 385 (1953).

to normal levels by the simultaneous injection of estradiol and progesterone. Alfert and Bern[109] report no change in the amount of DNA per nucleus in uterine cells during the estrus cycle. Rat seminal vesicles lost relatively more PNA than DNA following castration, but on treatment with testosterone the PNA and DNA contents and the PNA/DNA ratio finally exceeded the control values.[52] Castration and the subsequent injection of testosterone have relatively little influence on the nucleic acid content of liver in male and nonpregnant female rats[11]; nor has testosterone any influence on bone marrow composition.[186]

XI. Various Pathological Conditions

1. Pernicious or Megaloblastic Anemias

In such cases the nucleic acid content of human bone marrow shows the greatest changes from normal.[69] Both Davidson *et al.*[69] and Menten and Willms[187] find the average DNA-P content per nucleus increased to about twice the normal diploid amount per cell and the PNA/DNA ratio is raised above the normal level. Although there is some difference of opinion about the fall in DNA per cell on treatment with vitamin B_{12}, both groups find that the PNA/DNA ratio reduced as the bone marrow picture returns to normal.[69, 188]

2. Nerve Section, Ischemia, and Atrophy

Wallerian degeneration in the peripheral end of a nerve after section of the sciatic nerve in cats is accompanied by increasing PNA and DNA concentrations until the sixteenth day, and an increase in the PNA/DNA ratio from 0.9 to a peak value of 2.0 at 32 days when cellular proliferation is intense.[76] Similar changes occur after nerve crush. After nerve section and muscle atrophy, Mandel[85] reports that muscle weight falls by 60 %, PNA content by 55 %, and protein by 70 %.

Ischemia, after the ligation of the pedicle of the anterior left lobe of mouse liver, caused a 75 % reduction in PNA but not in DNA concentration after 24 hr.[189] After duct ligation, mouse submaxillary glands atrophy, causing a 73 % fall in PNA-P content but only a 30 % fall in DNA-P after 30 days.[71]

Radiation Effects

The irradiation of rats with X-rays at a dosage of 500 r. greatly reduced the concentrations of PNA and DNA in the bone marrow after 4 days; at

[186] C. Lutwak-Mann, *Biochem. J.* **52**, 356 (1952).
[187] M. L. Menten and M. Willms, *Arch. Pathol.* **54**, 343 (1952).
[188] M. L. Menten and M. Willms, *Arch. Pathol.* **54**, 351 (1952).
[189] P. Drochmans, *Experientia* **3**, 421 (1947).

the same time the PNA/DNA ratio was increased.[186, 190] In rat spleen[186] and mouse thymus[73] X-ray irradiation produced a large reduction in the PNA and DNA contents per organ. The exposure of rabbit bone marrow to γ-radiation reduced the concentrations of PNA and DNA but did not alter the average amount of DNA per nucleus in marrow cells.[191] The PNA per unit protein was decreased in the mitochondrial, microsome, and supernatant fractions of the cytoplasm in rabbit bone marrow, and in mouse spleen after X-ray irradiation.[192] It is suggested that irradiation causes a selective elimination of cell types with high concentrations of PNA.

4. Drug Action

The nitrogen mustard HN2 [methylbis(β-chloroethyl)-amine] prevents the normal increase of DNA in amphibian embryos.[193] Administration of HN2 to rats 1 hr. after hepatectomy caused a decrease in cellularity below control level at 36 hr., and at the same time raised the average DNA per cell by 19% and PNA per cell by 23%.[115] Ultmann *et al.* concluded that HN2 has a "temporary inhibitory action on mitosis when a single dose of the drug is given."

Injection of phenylhydrazine leads to the destruction of mature erythrocytes and the liberation into blood of immature reticulocytes, resulting in a 10- to 30-fold increase in nucleic acid (mostly PNA) concentration in blood.[75, 194] An anesthetic dose of sodium phenobarbitol increased the total nucleic acid content of spleen and reduced by almost half the PNA/DNA ratio.[61] The PNA, DNA, and total protein per liver increase appreciably 24 hr. after the injection of carbon tetrachloride into rats, probably as a result of proliferation of new cells in the periphery of the liver lobules.[195]

X. Plant Tissues

Very little information about the nucleic acid content of plants is available so far. Von Euler and Hahn[196] have shown that the PNA-P concentration decreases in rye-plants from the roots to the tip, and that it is lower in triploid than in diploid aspen. The ratio of PNA/DNA is 6.2 in the corn root tip meristem.[24]

Wildman *et al.*[197] applied the Schneider procedure to spinach leaves and

[190] P. Mandel, P. Metais, C. M. Gros, and R. Voegtlin, *Compt. rend.* **233,** 1685 (1951).
[191] J. F. Thomson, W. W. Tourtelotte, M. S. Carttar, and J. B. Storer, *Arch. Biochem. and Biophys.* **42,** 185 (1953).
[192] E. S. Maxwell and G. Ashwell, *Arch. Biochem. and Biophys.* **43,** 389 (1953).
[193] D. Bodenstein and A. A. Kondritzer, *J. Exptl. Zool.* **107,** 109 (1948).
[194] B. W. Halloway and S. H. Ripley, *J. Biol. Chem.* **196,** 695 (1952).
[195] R. M. Campbell and H. W. Kosterlitz, *Brit. J. Exptl. Pathol.* **33,** 518 (1952).
[196] H. von Euler and L. Hahn, *Arkiv Kemi, Mineral. Geol.* **25B,** 1 (1947).
[197] S. G. Wildman, J. M. Campbell, and J. Bonner, *Arch. Biochem.* **24,** 9 (1949).

found 32.5 % of the total P in the hot TCA extract of the tissue residue. According to Fries,[198] the NAP concentration per unit dry weight in the leaves of a variety of deciduous trees varies between 0.12 and 0.78 mg./g. dry weight. During discoloration of the leaves, the NAP concentration decreased by 20 to 40 %, and about 400 mg. nucleic acid eventually falls on each square mile of soil every year.

Following Pirie's observations[199] that the greatest yield of nucleoprotein came from the smallest leaves, Holden[200] applied a modification of the Ogur and Rosen[24] method to the leaves from tobacco plants and found that the concentration of both PNA and DNA was at least four times as high in the small as in the large leaves. There were three to four times the number of cells per unit area in the small leaves, and leaf size increased by cell enlargement and not by cell proliferation. Of the total phosphorus of the leaf, 30 % is PNA-P, two-thirds of which is located in the fiber. DNA-P accounts for 7 % and could not be detected in the chloroplasts.[200] Metzner,[201] using cytochemical methods, claims that both PNA and DNA are present in chloroplasts.

XI. Bacteria and Viruses

A number of reviews have appeared which deal particularly with the nucleic acid content of bacteria.[6, 202, 203] There is, however, not nearly as much detailed information about bacterial cell composition as is available for metazoan tissues.

According to Belozersky[203] the total nucleic acid concentrations (on a dry weight basis) of such bacteria as *Micrococci*, *Staphylococci*, and *E. coli* vary between 10 and 22 %, whereas for yeasts and various molds the concentrations are below 10 %. In general agreement with these values are Vendrely's[202] results, which also show that the PNA/DNA ratio ranges from 2 for *E. coli* to nearly 13 for baker's yeast. Mitchell and Moyle[204] report ratios of the same order for a large series of micro organisms.

It must be remembered, however, that the nucleic acids will vary in amount with the physiological state of the culture. The concentrations of PNA and DNA are reported to increase in rapidly growing yeast cells[205] and to reach a maximum just before the onset of cell multiplication in cul-

[198] N. Fries, *Plant and Soil* **4**, 29 (1952).
[199] N. W. Pirie, *Biochem. J.* **47**, 614 (1950).
[200] C. Holden, *Biochem. J.* **51**, 441 (1952).
[201] H. Metzner, *Naturwissenschaften* **39**, 64 (1952).
[202] R. Vendrely, "Un symposium sur les protéines," p. 165. Masson et Cie, Paris, 1946.
[203] A. N. Belozersky, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 1 (1947).
[204] P. Mitchell and J. Moyle, *Nature* **166**, 218 (1950).
[205] F. J. Di Carlo and A. S. Schultz, *Arch. Biochem.* **17**, 293 (1948).

tures of *E. coli*,[206] and during the rapid growth phase of *Micrococcus pyrogenes*[207]; following intensive fermentation, the PNA concentration per unit dry weight is increased in baker's yeast.[208]

A rise in the PNA/DNA ratio occurs during the early growth stages of *Staph. aureus*,[209] in *E. coli*,[206] and over the first 3 hr. growth of *Salmonella* and *Shigella* cultures.[210] In these last, the PNA per unit PN increased while the DNA per unit PN decreased over the first part of the growth phase. In the lag phase of *Staph. muscae* cultures, Price[211] reports a 60 % increase in PNA and protein content, and a 50 % increase in dry weight, without any change in cell count. DNA synthesis began only 45 min. after that of PNA and PN (cf. chick heart explants, Section V).

Caldwell and Hinshelwood[212] have carried out an interesting investigation into the actual content per cell during the growth of cultures of *B. lactis aerogenes*. Although the cell nitrogen content varies between 50 and 220 mg. N per 10^{12} cells, the DNA per cell remained constant at about 2 mg. DNA-P per 10^{12} cells. The PNA content fluctuated between values of 3 and 32 mg. PNA-P per 10^{12} cells. Colchicine did not alter the amount of DNA per cell, but it did increase the PNA/DNA ratio. During the normal growth of cultures,[213] the PNA-P per unit cell N *and per cell* rose and maintained a roughly linear relationship to the reciprocal of the mean generation time (i.e. the "growth rate"). Caldwell and Hinshelwood[214] have also proposed that "towards the end of the growth cycle when the medium becomes depleted of phosphorus, there is in fact an extensive conversion of PNA to DNA." In growing cultures of *B. lactis aerogenes*, the PNA-P and DNA-P account for 61 % and 18 %, respectively, of the total P content of the cells.

Complications can arise when the standard methods for determining PNA and DNA are applied to bacterial cells.[29, 31] In cultures of *Micrococcus pyogenes*, PNA and DNA account for 29 % and 11 % of the total P.[29] Sherratt and Thomas[31] report that the "bound DNA" (insoluble in alkaline digest III, Table I) has 22 % excess P, while the PNA fraction contains 15 % excess P. All these fractions reached their maximal amount per cell during the logarithmic phase of growth.

According to Mitchell and Moyle,[207] the PNA concentration in cells of *Micrococcus pyogenes* appears to control the rate of growth. Addition of

[206] M. L. Morse and C. E. Carter, *J. Bacteriol.* **58**, 317 (1949).
[207] P. Mitchell and J. Moyle, *J. Gen. Microbiol.* **5**, 421 (1951).
[208] H. von Euler and L. Hahn, *Arkiv Kemi, Mineral. Geol.* **26A**, 10pp. (1948).
[209] C. A. Fish, I. Asimov, and B. S. Walker, *Proc. Soc. Exptl. Biol. Med.* **75**, 774 (1950).
[210] T. Brechbuhler, *Bull. soc. chim. biol.* **32**, 952 (1950).
[211] W. H. Price, *J. Gen. Physiol.* **35**, 741 (1952).
[212] P. C. Caldwell and Sir Cyril Hinshelwood, *J. Chem. Soc.* **1950**, 1415.
[213] P. C. Caldwell, E. L. Mackor, and Sir Cyril Hinshelwood, *J. Chem. Soc.* **1950**, 3151.
[214] P. C. Caldwell and Sir Cyril Hinshelwood, *J. Chem. Soc.* **1950**, 3156.

penicillin to a culture of these bacteria decreased the absolute rate of PNA synthesis more than that of DNA. These authors suggest that "the rate of cell protein synthesis appears to be controlled by the percentage weight of nucleic acids in the cells, while the nucleic acid synthesis appears to be more directly determined by the nutritive properties of the medium than protein synthesis."

From his studies on continuous cultures of *Polytomella caeca*, Jeener[215] has developed the very important concept that "the rate of increase of cellular proteins is proportional to the quantity of RNA which is in excess of a basal quantity concerned with the renewal of the existing protein." Only in this way could he account for the unexpected decrease in the amount of PNA per unit protein which occurred in cultures when cell multiplication and, therefore, protein synthesis, was slowed down by limitation of either phosphate or organic nutrients, such as ethanol and acetate.

Gale and Folkes[216a, b] also report a strong positive correlation between the rate of protein synthesis and the nucleic acid content of the cells, in this case, *Staphylococcus aureus*. Although chloramphenicol, aureomycin, and terramycin completely inhibited protein synthesis in this organism, they stimulated the rate of formation of nucleic acid.

Dirx[217] found that the PNA content of the spores in a variety of molds ranges from 0.145 pg. per spore for *Penicillium* to 1.93 pg. for *Aspergillus*. In yeast cells, Ogur *et al.*[41] report that the DNA-P per cell increases in direct ratio to the degree of ploidy; so do dry weight, PNA, and metaphosphate, but with a much wider experimental variation.

Little can be said about the nucleic acid composition of viruses. Reviews are available by Knight,[218] Beard,[219] and Davidson.[6] Both PNA and DNA are found in viruses, but, as Knight pointed out, only PNA has been observed so far in plant viruses, whereas either PNA or DNA or both are present in animal viruses. In plant viruses (e.g., tobacco mosaic, tomato bushy stunt, and southern bean mosaic) the PNA concentration ranges from 6 to 40 % on a dry weight basis, whereas for a few animal viruses it is always below 10 %. The DNA concentration varies between 2 % in influenza virus[218] to 40 % in T_2 bacteriophage,[220] each of which contain some PNA.

XII. Conclusions

Certain definite associations between the nucleic acids and tissue metabolism can be seen in the results described in this review. Whereas changes

[215] R. Jeener, *Arch. Biochem. and Biophys.* **43,** 381 (1953).
[216] (a) E. F. Gale and J. P. Folkes, *Biochem. J.* **53,** 483 (1953); (b) *ibid.* p. 493.
[217] J. Dirx, *Biochim. et Biophys. Acta* **8,** 196 (1952).
[218] C. A. Knight, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 115 (1947).
[219] J. W. Beard, *Physiol. Revs.* **28,** 349 (1948).
[220] A. Taylor, *J. Biol. Chem.* **165,** 271 (1946).

in DNA content are always linked to changes in cell number, the amount of PNA in a tissue depends on its physiological function or state, high concentrations being associated with cell populations which are actively dividing or actively synthesizing protein. Although the proportion of PNA to protein is remarkably similar for a number of different tissues, it is clear that PNA and protein can vary independently around the normal basic level. A higher proportion of PNA to protein than is present in adult or resting cell populations is found in embryonic, regenerating, and neoplastic tissues, and in rapidly dividing bacterial populations. Increased energy intake and treatment with cortisone, thyroxine, estradiol, and insulin will also raise the proportion of PNA to protein. Protein and vitamin C-deficient diets produce a similar effect but act by decreasing the protein to a greater extent than the PNA. Conditions which predominantly reduce the PNA and, hence, the proportion of PNA to protein include a high fat diet and thiamine and vitamin B_{12} deficiencies.

In conclusion, variations in PNA content are produced primarily by changes in energy intake and energy metabolism and, of course, by factors directly affecting nucleic acid synthesis. Secondly, increases in the proportion of PNA to protein above the normal adult or resting level invariably precede protein synthesis and cell multiplication.

XIII. Addendum

An interesting development in the ultramicrotechniques for determining the PNA and the nucleotide content of individual cells has been described by Edström.[221] He has also developed a method of determining the amounts of individual nucleotides in the PNA from a single cell, involving their separation by ionophoresis on a copper-silk fiber.[222] While previous ionophoretic and chromatographic techniques measure quantities of the order of 100 to 1000 μg. PNA, these methods have been successfully applied to nerve cells containing 200 to 1000 pg. PNA. Motor anterior horn and spinal ganglion cells from the rabbit contained on the average between 550 and 560 pg., although the concentrations (mass/cell volume) were 2.4 and 1.1 %, respectively.

In their studies on the embryonic development of the sea-urchin, Elson *et al.*[223] measured the PNA content by summation of the amounts of individual pentose nucleotides and found that the PNA values by orcinol determinations were on the average 8 % less, while those from phosphorus determinations were 10 % more, than the total nucleotide results. The PNA content fluctuated within the range 5 to 6 $\times$ 10^{-3} μg per embryo

[221] J.-E. Edström, *Biochim. et Biophys. Acta* **12**, 361 (1953).
[222] J.-E. Edström, *Nature* **172**, 809 (1953).
[223] D. Elson, T. Gustafson, and E. Chargaff, *J. Biol. Chem.* **209**, 285 (1954).

for 60 hours after fertilization, while the DNA content doubled every hour in the first 10 hours, and at 10 and 40 hours indicated that there were 900 and 3550 cells per embryo, respectively. Using an isotopic dilution method to measure DNA and a chromatographic method for PNA, Marshak and Marshak[224] find that *Arbacia* eggs contain 24.4×10^{-4} μg. PNA per egg and 8.1×10^{-6} μg. DNA per egg. In human adrenal tissue, the PNA-P and DNA-P concentrations are 20.5 to 41.3 and 12.2 to 18.5 mg./100 g. tissue, respectively.[225] The PNA and DNA fractions in *Mycobacterium tuberculosis* are reported to contain 29.8% and 6.0% of the total phosphate content of the organisms.[226] Vendrely and Tulasne[227] find that the dwarf, or L-forms of *Proteus* (P 18) have an average PNA/DNA ratio of 0.14 as compared with the ratio of 2.50 in the normal forms.

A warning of the danger of applying the Schmidt and Thannhauser method to all tissues without preliminary investigation has been given by Drasher.[228] Her results with mammary tumor tissue from C3H mice led her to conclude that a significant amount of the DNA was being hydrolyzed by the alkaline digestion and was passing into the PNA fraction, thus producing a much higher PNA-P/DNA-P ratio for the Schmidt and Thannhauser than for the Schneider method (see also footnote *c*, Table II).

The DNA content of tissues is being used increasingly as a means of determining changes in cell number in growing tissues. In the virgin mouse uterus, Drasher[229] found that the total DNA content or cell number increased most rapidly between 2½ and 6 months, and that the PNA/DNA ratio was higher in the stimulated stages (proestrus, estrus, and early metestrus) than in the unstimulated stages (late metestrus and diestrus). Nowinski and Yushok[230] measured the DNA content per nucleus in wings and legs of 5- to 12-day-old chick embryos by the Schneider method and obtained considerably higher average values than those previously reported[231-233] for chick embryonic cells. The time required to enable the DNA content to double in amount varied from 25 hours for legs in the 6- to 7-day embryos to 31 hours for both legs and wings in the 11-day embryos.[230]

[224] A. Marshak and C. Marshak, *Exptl. Cell Research* **5,** 288 (1953).
[225] J. N. Davidson, unpublished results.
[226] F. Winder and J. M. Denneny, *Nature* **174,** 353 (1954).
[227] R. Vendrely and R. Tulasne, *Nature* **171,** 262 (1953).
[228] M. L. Drasher, *Science* **118,** 181 (1953).
[229] M. L. Drasher, *Proc. Soc. Exptl. Biol. Med.* **84,** 596 (1953).
[230] W. W. Nowinski and W. D. Yushok, *Biochim. et Biophys. Acta* **11,** 497 (1953).
[231] J. N. Davidson, I. Leslie, R. M. S. Smellie, and R. Y. Thomson, *Biochem. J.* **46,** xl (1950).
[232] A. E. Mirsky and H. Ris, *Nature* **163,** 666 (1949).
[233] W. E. J. Phillips, W. A. Maw, and R. H. Common, *Can. J. Zool.* **31,** 167 (1953).

In view of discrepancies between the results of Nowinski and Yushok and those of other authors for DNA in chick embryonic nuclei, it should be mentioned that good agreement has been obtained for determinations of DNA made on replicate roller tubes of chick heart explants by the Schmidt and Thannhauser procedure and the microbiological assay for deoxyribosides of Hoff-Jørgensen.[234]

Patterson and her colleagues,[235] in their studies on salivary glands of *Drosophila melanogaster*, report that females of genotypes *XX* and *XXY* have significantly higher amounts of PNA per gland and PNA per cell than males of genotypes *XO* and *XY*, but that there are no significant differences within the male and female pairs. The *Y* chromosome in this case has no measurable effect on the amounts of nucleic acids in the salivary gland cells.

In the regenerating mouse liver,[236] the restoration of DNA was completed after 8 days, but only 60% of the original number of nuclei were present. While the ratio of DNA/protein was remarkable constant over 28 days' regeneration, the PNA/protein was greater than normal values as long as protein synthesis was proceeding. Einhorn *et al.*[237] have studied the action of cortisone on regenerating liver, and confirm previous findings by Roberts *et al.*[168] In both normal and regenerating liver, cortisone treatment produced decreased cellularity and inhibited the restoration of DNA to a greater extent than PNA.

It is not possible to summarize adequately the detailed account given by Laird[238] of the proportions of PNA, DNA, and protein found in the different intracellular fractions of a number of normal and malignant tissues from rat, mouse, and man. Using the Schneider method, she has found higher values for the DNA per average nucleus than previously reported. Distinctly different patterns of intracellular distribution were apparent amongst the various tissues; in particular, there were smaller amounts of cytoplasmic particulate fractions in adrenal, thymus, and all tumors than in liver, kidney, pancreas, and submaxillary gland: in tumor mitochondria the PNA/protein ratio was, however, higher than in any of the normal tissues. All the tumors examined were closely similar in the proportion and composition of their intracellular fractions. Albert and Johnson[239] confirm that the lower PNA-P content of the cytoplasmic

[234] E. Hoff-Jørgensen and I. Leslie, unpublished results.

[235] E. K. Patterson, Helga M. Lang, M. E. Dackerman, and J. Schultz, *Exptl. Cell Research* **6**, 181 (1954).

[236] K. K. Tsuboi, H. O. Yokoyama, R. E. Stowell, and M. E. Wilson, *Arch. Biochem. and Biophys.* **48**, 275 (1954).

[237] S. L. Einhorn, E. Hirschberg, and A. Gellhorn, *J. Gen. Physiol.* **37**, 559 (1954).

[238] A. K. Laird, *Exptl. Cell Research* **6**, 30 (1954).

[239] S. Albert and R. M. Johnson, *Cancer Research* **14**, 271 (1954).

articles in tumor cells is accompanied by higher PNA-P/N values in both these particles and the supernatant fraction (see also ref. 159, Table IX).

Klein, Rasch, and Swift[240] find that during the production of tumors in the stems of the broad bean with *Agrobacterium rubi*, the DNA concentration increased to a peak with little change in PNA concentration and intensive cell division occurred; with indoleacetic acid, on the other hand, there was a 50% increase in PNA concentration in the first 2 days, and a decline in DNA concentration, accompanying the enlargement of cells. Indoleacetic acid(IAA) was also used by Silberger and Skoog[241] to induce changes in cultures of pith tissue of *Nicotiana tabacum* stems. The higher concentrations (1.4 mg./l.) of IAA caused cell enlargement and a greater increase in PNA than DNA. With much lower concentrations, DNA was preferentially increased, and cell division was in evidence.

When the DNA content was measured in the cells of the DBA mouse ascites thymoma, Levy *et al.*[242] found that the peak in the DNA content per nucleus coincided with peak values for PNA per cell. Klein and Forssberg[243] report reasonably good agreement between the Schmidt and Thannhauser and Schneider methods, when applied to the Ehrlich ascites tumor. Following irradiation of mice with 1250 r., there was a nearly linear increase in the PNA and protein per cell, without any corresponding synthesis of DNA; the cell volume increases and the DNA concentration decreases during the period in which mitotic figures disappear.

Papers by Mandel and his colleagues cover a variety of topics. Continuing their studies on the action of X-rays on spleen and bone marrow, they find that a single dose of 700 r. causes a greater reduction in the PNA and DNA content per spleen than the same dosage given in two fractions at a 7-day interval.[244] In groups of rats injected with 0.5% alloxan solution, there are 16.7 and 18.1% reductions in the DNA and PNA content of the pancreas, respectively, 2 days after administration, and smaller and less-prolonged reductions in liver PNA and DNA.[245] On the other hand, Bass *et al.*[246] report that the increase in DNA content per liver which occurred in their alloxan diabetic rats was a direct effect of the alloxan on the liver cells, and was not a consequence of the diabetic state. In a more detailed account of earlier work (ref. 173, Table X) on the influence of insulin on

[240] R. M. Klein, E. M. Rasch, and H. Swift, *Cancer Research* **13,** 499 (1953).

[241] J. Silberger, Jr., and F. Skoog, *Science* **118,** 443 (1954).

[242] H. B. Levy, H. M. Davidson, R. W. Reinhart, and A. L. Schade, *Cancer Research* **13,** 716 (1953).

[243] G. Klein and A. Forssberg, *Exptl. Cell Research* **6,** 211 (1954).

[244] C. M. Gros, P. Mandel, and J. Rodesch, *Compt. rend. soc. biol.* **147,** 1279 (1953).

[245] J. D. Weill, P. Mandel, and O. Zalis, *Compt. rend. soc. biol.* **147,** 1288 (1953).

[246] A. D. Bass, H. F. Diermeier, H. S. Di Stefano, and E. J. Cafruny, *J. Pharmacol. Exptl. Therap.* **107,** 478 (1953).

the growth of embryo chick heart explants, Leslie and Paul[247] report that increased synthesis of phospholipids, PNA, and DNA occurred at concentrations of 1×10^{-2} to 3 units insulin/ml.; the response was not caused by the presence of hyperglycemic factor.

During the atrophy of the gastrocnemius muscle after section of the sciatic nerve, Mandel, Bieth, and Weill[248] find that changes in the inorganic and ester phosphate follow the same pattern when results are expressed in amounts per muscle and in amounts relative to the DNA-P content. In another study, when oxygen uptake of rat tissue slices was referred to fresh weight, kidney was most active, followed by brain, spleen, and liver; when expressed as mm.^{3} O_2/hr./unit DNA, brain had twice the uptake of kidney, three times that of liver, and about ten times that of spleen.[249]

Mandel, Metais, and Voegtlin[250] determined the PNA, DNA, and protein content of the bone marrow of the long bones of rats after depriving the animals of one or other of their endocrine glands. Expressing results as amounts per 100 g. body weight, they found that thyroidectomy reduced protein, PNA, and DNA in that order, that gonadectomy had little effect on male, but reduced PNA, DNA, and protein in female bone marrow, that adrenalectomy reduced all three constituents by 20 to 30% in 3 to 5 days, while hypophysectomy increased their amounts in bone marrow over 28 days. In a continuation of previous work on the estrogen action on the immature pullet, Phillips *et al.*[233] found that testosterone propionate and estradiol benzoate (singly or combined) did not alter the average DNA-P content per nucleus of the liver (see footnotes 39, 180). Rat mammary gland[251] shows low values for the total content of PNA, DNA, and PNA/DNA in the virgin animal; during pregnancy DNA rises to a plateau level at 10 days, while PNA and total N continue to increase to a maximum throughout pregnancy and most of lactation, producing a peak PNA/DNA ratio of 3.

When different groups of human bone marrow samples (normal, iron-deficiency anemia, untreated megaloblastic anemia, and untreated leukemias) were examined, White *et al.*[252] found that within each series the DNA-P content per cell was constant over a wide range of marrow cellularity. Only in the case of the megaloblastic anemia was the PNA-P per cell significantly higher than in normal cells. Menten *et al.*[253] and Menten

[247] I. Leslie and J. Paul, *J. Endocrinol.* **11,** 110 (1954).
[248] P. Mandel, R. Bieth, and J. D. Weill, *Bull. soc. chim. biol.* **35,** 973 (1953).
[249] M. Jacob, L. Mandel, and P. Mandel, *Compt. rend. soc. biol.* **147,** 1276 (1953).
[250] P. Mandel, P. Metais, and R. Voegtlin, *Compt. rend. soc. biol.* **147,** 1282 (1953).
[251] W. R. Kirkham and C. W. Turner, *Proc. Soc. Exptl. Biol. Med.* **83,** 123 (1953).
[252] J. C. White, I. Leslie, and J. N. Davidson, *J. Pathol. Bacteriol.* **66,** 291 (1953).
[253] M. L. Menten, M. Willms, and L. D. Wright, *Cancer Research,* **13,** 729 (1953).

and Willms[254] have measured the PNA and DNA in splenic lymphocytes of the G57 strain of black mice, and in samples of bone marrow from leukemic patients, and propose on the basis of rather few results, that there are a series of 'fixed relationships' for the ratios PNA-P/DNA-P in the various cells.

Although fresh-water planarian worms contained only 14% of their original total nitrogen after 60 days' starvation, there was no significant change in their DNA content.[255] An earlier report by Logan[256] that increases in PNA and DNA occurred in the proximal as well as the distal portion of a crushed sciatic nerve has been supported by the confirmation of increased cellularity in both segments.[257] Protein depletion[258] and thiamine deficiency[259] produced similar effects on the total nucleic acid concentrations in the sciatic nerve, although the authors have complicated interpretation by introducing weight controls (younger animals of same weight as animals on deficient diet) in addition to the usual "age" controls. The deficient diets had little or no influence on the nucleic acid concentrations if comparison was made with age controls.

Hypoxia, produced by an oxygen concentration of 7.75%, caused only slight increases in DNA concentration of rat bone marrow and spleen, but the spleen weight increased by 175%.[260] By growing *E. coli* and *Clostridium welchii* in normal and magnesium-deficient media, Webb[261] has been able to show that DNA remains constant in amount in the normal free cells (2.32×10^{-3} pg./cell) or in the unit cells of the filamentous forms occurring in Mg-deficient medium (2.36×10^{-3} pg./cell). He also found lower ratios of acid-soluble nitrogen and protein nitrogen to the DNA-P in the Mg-deficient organisms.

Fonnesu and Severi[262] experimentally produced "cloudy swelling" of kidney cells by injecting Wistar strain rats with *S. typhimurium* toxin or mercuric chloride, or by ligaturing the renal pedicle, and showed that the histological changes were accompanied by a decrease in the total amounts of DNA per kidney. Suzuki[263] treated rabbits with methylthiouracil and

[254] M. L. Menten and M. Willms, *Cancer Research* **13,** 733 (1953).

[255] E. Hoff-Jørgensen, E. Løvtrup, and S. Løvtrup, *J. Embryol. Exptl. Morphol.* **1,** 161 (1953).

[256] J. E. Logan, *Can. J. Med. Sci.* **30,** 457 (1952).

[257] J. E. Logan, R. J. Rossiter, and M. L. Barr, *J. Anat.* **87,** 419 (1953).

[258] W. A. Mannell and R. J. Rossiter, *Brit. J. Nutrition* **8,** 44 (1954).

[259] W. A. Mannell and R. J. Rossiter, *Brit. J. Nutrition* **8,** 56 (1954).

[260] W. A. Rambach, J. A. D. Cooper, and H. L. Alt, *Science* **119,** 380 (1954).

[261] M. Webb, *Science* **118,** 607 (1953).

[262] A. Fonnesu and C. Severi, *Brit. J. Exptl. Pathol.* **34,** 341 (1953).

[263] N. Suzuki, *Japan. J. Physiol.* **3,** 279 (1953).

found over a 60-day period, a 119% increase in DNA-P/thyroid and a 210% increase in PNA-P/thyroid. The feeding of the insecticide aldrin (hexachlorohexahydrodimethanonaphthalene) to mice over 13 to 25 days, produced an enlargement of liver parenchyma, accompanied by a much greater rise in DNA content than in PNA content; the brain tissue showed no comparable changes under the same treatment.[264]

[264] E. Annau, *Can. J. Biochem. Physiol.* **32,** 178 (1954).

CHAPTER 17

Cytochemical Techniques for Nucleic Acids*

HEWSON SWIFT

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I. Introduction

Cytochemistry seeks to interpret the biochemistry of cells in terms of their morphology. A great deal of the current interest in the nucleic acids

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arises from their intimate association with genes and chromosomes, and their apparent involvement in protein synthesis. Cytochemical techniques have played a dominant part in establishing these relations.

All cytochemical reactions have their limitations and it is important to investigate these as fully as possible. It should be remembered that, at least for the present, relatively inaccurate or nonspecific methods may often serve a useful purpose, particularly where the variables are of known magnitude. With many biological problems, knowing whether the amount of nucleic acid in a cell increases or decreases is fully as important as knowing the exact amount present.

Cytochemistry, in treating the cells themselves as units, can supply information on individual cell variation that is obviously lost in the homogenates often used in biochemical procedures. It thus constitutes practically the only approach to many biological problems, for example, concerning the chemical changes that accompany mitosis. The three major cytochemical methods for nucleic acid localization depend on the three major nucleotide components. Basic dyes are associated with the phosphoric acid, the Feulgen reaction with the sugar, and ultraviolet absorption with the purines and pyrimidines.

II. Basic Dyes

It has been known for many years that the ability of fixed cells to bind basic dyes was associated, at least in part, with their nucleic acid content. Only comparatively recently, however, has there been any concerted attempt to use dye binding as a technique for the cytochemical study of nucleic acids. Among the early important studies were those of Mathews,[1] Hammarsten and Hammarsten,[2] and Brachet.[3] Recently basic dyes have been used in a large number of investigations, some in conjunction with microphotometric determinations. Variation in dye intensity has been interpreted as an indication of variation in nucleic acid concentration, or in some cases of "polymerization." To evaluate these techniques it is necessary briefly to examine the major factors affecting dye-nucleic acid interaction.

Without doubt the most important factor in dye binding is the electrostatic charge on the dye molecule and on the nucleoprotein with which it combines. Basic dyes are usually the salts of colored bases, and their common combining group, or auxochrome, is the amino group which exists in a positively charged dissociated form ($-NH_3^+$) except at high pH levels.

[1] A. Mathews, *Am. J. Physiol.* **1,** 445 (1898).

[2] E. Hammarsten, G. Hammarsten, and T. Teorell, *Acta Med. Scand. Suppl.* **196,** 1 (1928).

[3] J. Brachet, *Compt. rend. soc. biol.* **133,** 88 (1940).

The primary phosphoryl groups of nucleic acid have a pK of about 2,[4] and consequently are negatively charged at pH levels above 2. Dye-nucleate complexes are formed then, at least in large part, through salt linkages between cation and anion. Evidence for this view can be found in the fact that other cations, La^{+++}, Th^{++++}, protamines, histones, and fibrinogen, compete with the dye for binding sites on the nucleic acid.[5-8] Also, osmotic[9] and membrane potential studies[10,11] demonstrate that simple cations such as sodium may be adsorbed by nucleic acids in solution also through ion-pair formation. Such ions would be expected to compete in some cases with dyes for binding sites. For example, approximately a 20-fold decrease in quinoline binding by PNA in solution was brought about by a 10-fold increase in the concentration of sodium chloride or magnesium sulfate.[12]

There have been several studies of dye binding by nucleic acids in solution. Stoichiometric precipitation of dye-nucleate has been reported with crystal violet[13,14] and toluidine blue.[8] Under certain conditions nucleic acid depolymerization resulted in decreased binding of acridines,[15,16] quinolines,[12] methyl green, ethyl green, and malachite green,[14,17] although methyl green was bound stoichiometrically to polymerized DNA.[6] On the other hand, nucleic acid depolymerization increased the uptake of pyronin Y[14] and rosaniline,[18] but had no effect on the degree of binding of Victoria blue, crystal violet,[14] methylene blue,[19] or sodium ions.[11] It has been suggested that the decrease in binding with depolymerization may be associated with altered steric[14,20] or electrostatic[16] conditions of the nuelcic acid molecule. The increase in binding shown with pyronin and rosaniline may involve the removal of steric hindrance, alteration in the configuration of charge centers, or the opening up of new binding sites.[18]

Other factors in addition to electrostatic charge are undoubtedly of im-

[4] D. O. Jordan, *Progr. Biophys.* **2**, 51 (1952).
[5] E. G. Kelley, *J. Biol. Chem.* **127**, 55 (1939).
[6] N. B. Kurnick and A. E. Mirsky, *J. Gen. Physiol.* **33**, 265 (1950).
[7] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **34**, 475 (1951).
[8] H. Herrmann, J. S. Nicholas, and J. K. Boricious, *J. Biol. Chem.* **184**, 321 (1950).
[9] E. Hammarsten, *Biochem. Z.* **144**, 383 (1924).
[10] J. M. Creeth and D. O. Jordan, *J. Chem. Soc.* **1949**, 1409.
[11] J. Shack, R. J. Jenkins, and J. H. Thompsett, *J. Biol. Chem.* **198**, 85 (1952).
[12] F. S. Parker, *Science* **110**, 426 (1949).
[13] R. Feulgen, *Z. physiol. Chem.*, **84**, 309 (1913).
[14] N. B. Kurnick, *J. Gen. Physiol.* **33**, 243 (1950).
[15] J. L. Irwin and E. M. Irwin, *Science* **110**, 426 (1949).
[16] J. L. Irwin and E. M. Irwin, *Federation Proc.* **11**, 235 (1952).
[17] N. B. Kurnick, *Arch. Biochem.* **29**, 41 (1950).
[18] L. F. Cavalieri and A. Angelos, *J. Am. Chem. Soc.* **72**, 4686 (1950).
[19] R. Vercauteren, *Nature* **165**, 603 (1950).
[20] R. Vercauteren, *Enzymologia* **14**, 134 (1950).

portance. In binding between fatty acids and protein basic groups, Boyer, Ballou, and Luck[21] showed that the length of the carbon chain was an important factor. Larger molecules have increased binding energy, possibly through their greater ability to release bound water molecules[22] although the initial binding force is electrostatic. In studies on methyl orange-protein binding, Klotz and Urquhart[23] showed that buffer anion competition was in general larger the larger the ion. Such factors are doubtless also of importance in determining the dissociation constants of dye-nucleate complexes. In some cases proteins have been shown to bind un-ionized compounds, in which case nonelectrostatic forces must obviously be involved.[24,25] Binding of this type may possibly be seen in nonspecific adsorption of dye by tissue sections (see below). With some dyes, for example, toluidine blue, this may be negligible after proper differentiation. Under similar conditions, however, nonspecific adsorption of other dyes, such as crystal violet, or basic fuchsin, may be intense. Van der Waals forces are apparently responsible for the formation of dye complexes that occur in more concentrated aqueous solutions of many dyes. This phenomenon is associated with dye metachromasy, and is discussed below.

1. Specificity

As has frequently been pointed out, there are other acidic groups in tissues besides nucleic acid, capable of binding basic dyes. Protein carboxyl groups bind dyes at high pH. This has been shown, for example, by Singer and Morrison[26] in the case of fibrin films. Bartholomew, Evans, and Nielson[27] showed that basic dye binding was decreased at pH 9 and 11.5 when carboxyl groups were blocked by methylation. Since the pK of protein carboxyl groups is around 5, at pH levels below this carboxyl binding by most basic dyes is negligible, for example, in tissue proteins after nucleic acids have been removed by treatment in hot trichloroacetic acid or enzymes.[8,28] This is also evident in Fig. 1, showing nucleic acid and protein staining with azure B. In the pH range from about 3 to 5, basophilia in most cases is due entirely to nucleic acid. Above pH 5, basophilia is also due to carboxyl binding.

The mucin of goblet cells, mast cell granules, and the chondroitinsulfuric acid of cartilage are also strongly basophilic, presumably because such

[21] P. D. Boyer, C. A. Ballou, and J. M. Luck, *J. Biol. Chem.* **167,** 407 (1947).
[22] I. Klotz, *Cold Spring Harbor Symposia Quant. Biol.* **14,** 97 (1950).
[23] I. Klotz and J. M. Urquhart, *J. Am. Chem. Soc.* **71,** 1597 (1949).
[24] I. Klotz and J. Ayers, *Federation Proc.* **11,** 240 (1952).
[25] G. M. Allenby and H. B. Collier, *Arch. Biochem. and Biophys.* **38,** 147 (1952).
[26] M. Singer and P. H. Morrison, *J. Biol. Chem.* **175,** 133 (1948).
[27] J. W. Bartholomew, E. E. Evans, and E. D. Nielson, *J. Bacteriol.* **58,** 347 (1949).
[28] M. Flax and A. W. Pollister, *Anat. Record* **105,** 56 (1949).

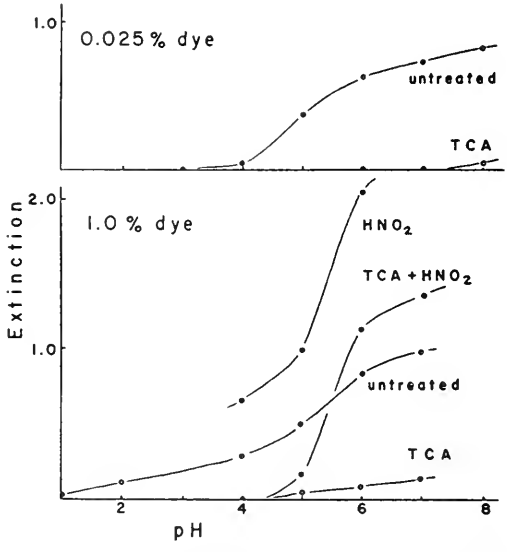


FIG. 1. The effect of pH and ionized groups on basic dye binding. The extent of carboxyl group binding is evident when nucleic acids are removed by trichloroacetic acid. Partial removal of amino groups with nitrous acid results in a marked increase in dye binding by both phosphoryl and carboxyl groups. Acetic alcohol-fixed mouse liver serial sections, cut at 6 μ and stained for 1 hour at 30° in azure B solution; rinsed for 1 hour in water at the same pH as staining bath, dehydrated with *tert*-butyl alcohol, and mounted in clarite; pH adjusted with 0.1 *N* HCl or NaOH; 5% TCA treatment, 15 minutes at 90°; HNO_2 treatment, 15% $NaNO_2$ in 3 parts 50% ethanol to 1 part glacial acetic acid at room temperature for 1 hour. Each point represents the mean of 5 or 10 measurements of areas 25 μ in diameter made at a wavelength of 590 mμ. Points are approximately equivalent to amounts of dye bound, but are too low at higher extinctions due to the influence of metachromasy.

compounds contain esters of sulfuric acid.[29] Such compounds are not removed by ribonuclease or hot trichloroacetic acid. They also stain a different color than PNA with many basic dyes (e.g., the thiazines) and, since they are usually associated with polysaccharides, may also be distinguished by comparison with adjacent serial sections on which the periodic acid-Schiff reaction has been performed.[30] The phosphoproteins of yolk may also be basophilic, but staining is not diminished by ribonuclease.[31] In general, basophilia due to such compounds, then, need not be confused with nucleic acid staining.

We can conclude that basic dye binding, if carried out at pH values near 4, is usually specific for nucleic acid. Control slides, however, should be

[29] L. Lison, *Arch. biol. (Liége)* **46**, 599 (1935).
[30] R. D. Lillie, *Anat. Record* **103**, 611 (1949).
[31] J. Brachet, *Quart. J. Microscop. Sci.* **94**, 1 (1953).

run, in which nucleic acid has been removed with ribonuclease, deoxyribonuclease, or acid treatment. These methods should distinguish other types of binding, such as that due to sulfuric acid esters, phosphoproteins, or the "nonspecific" background binding usually associated with inadequately differentiated slides (see below).

2. Protein Interference and Effect of pH

Basic dye binding by nucleic acids in tissue sections involves a complex equilibrium between many different factors. It is doubtful if all nucleic acid phosphoryl groups are ever active in dye binding. Some may be tied to the denatured tissue proteins, since without this or other linkage the lower polymers, at least, would be expected to be removed during certain steps in the process of histological preparation. The extent to which the dye can "compete" with protein basic groups in a fixed tissue is not clear. The situation is obviously different from the *in vitro* competition shown between dyes and proteins in solution. Protein inhibition can be made complete in tissue sections, so that staining is abolished, by incubating slides for a short time in protein solutions.[32]

Proteins, whether bound directly to nucleic acids or not, contain a large number of ionizable groups which can obviously alter electrostatic fields in their vicinity. The tremendous effect that protein basic groups have upon nucleic acid binding may readily be seen by study of the effects of blocking agents. Amino groups may be acetylated on slides by treating them with 100% acetic anhydride for 1 hour at room temperature[33] or may be blocked with a solution of chloramine-T.[34] The amino groups may be partially removed with nitrous acid (15% sodium nitrite in 3 parts of 50% ethanol to 1 part glacial acetic acid).[33] After such treatments nucleic acid binding may be increased several times (Fig. 1), and areas that were practically unstained before treatment may be strongly colored, as shown by Alfert[33] for methyl green.

The presence of protein amino groups is almost certainly responsible for modifying the effect of pH on nucleic acid binding. As can be seen from the data of Herrman, Nicholas, and Boricious[8] graphed in Fig. 2, toluidine blue adsorption of PNA in solution was found to be practically constant from pH 3.2 to 6.8 (curve *A*). With the same concentration of dye and nucleic acid, the addition of fibrinogen gave the values shown by curve *B*, and the binding of dye by muscle fibers by curve *C*. At pH 6.8 binding of muscle fibers was greater than that of pure nucleic acid solutions presumably because of the added effects of carboxyl binding. At lower pH the

[32] J. B. French and E. V. Benditt, *Federation Proc.* **11**, 415 (1952).
[33] M. Alfert, *Biol. Bull.* **103**, 145 (1952).
[34] L. Monné and D. B. Slautterback, *Arch. Zool.* [2] **1**, 455 (1951).

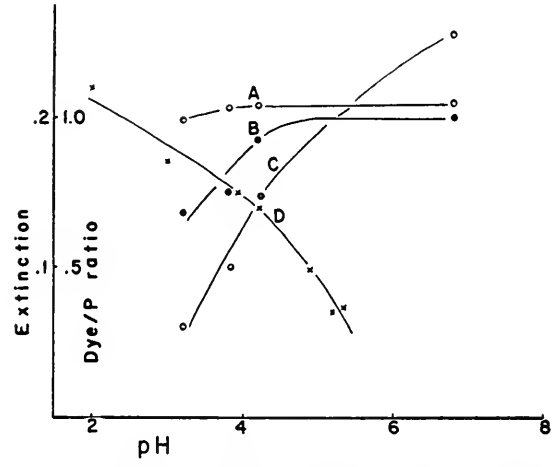


FIG. 2. The influence of proteins on basic dye binding. *A*. Dye/phosphorus ratio in precipitate from 1 ml. of 0.1% solution of PNA added to 9 ml. of 10^{-3} *M* toluidine blue. *B*. Same, with 0.1% fibrinogen added. *C*. Dye/phosphorus ratio in muscle of 17-day rat embryo stained with 10^{-3} *M* toluidine blue. Protein basic groups depress dye bending at lower pH; carboxyl binding is apparently responsible for the increased staining at pH 6.8. *D*. Orange G (10^{-5} *M*) binding by fibrin films, as an indication of the increasing charge of protein basic groups with decreasing pH. [*A*, *B*, and *C* from Herrmann, Nicholas, and Boricious[8]; *D* from Singer and Morrison[26].]

presence of protein basic groups has apparently decreased the basic dye adsorption. As one indication of the increased charge with decreasing pH shown by protein basic groups, the data of Singer and Morrison[26] for orange G binding of fibrin films is shown in curve *D*. This curve is roughly characteristic of a wide variety of proteins. The increase of acid dye binding with decrease in pH might not be expected, since the amino, guanidyl, and imidazole groups should be maximally ionized below pH 7. Apparently these groups are not always free to react, possibly because they are blocked by internal hydrogen bonding between amino and hydroxyl or carboxyl groups in the protein molecule, as suggested by Klotz.[22]

The equilibrium between unbound dye and the dye bound to a tissue section is thus influenced by pH through its action on the total electrostatic charge on the nucleoproteins of the tissue. Other factors can obviously shift the equilibrium in one direction or another. The role of these variables in textile dyeing has been discussed by Vickerstaff,[35] and their influence, largely as applied to protein binding, has been reviewed by Singer.[36]

[35] T. Vickerstaff, "The Physical Chemistry of Dyeing". Imperial Chemical Industries, London, 1950.

[36] M. Singer, *Intern. Rev. Cytol.* **1**, 211 (1952).

As shown by many workers,[26,37] an increase in dye concentration will increase the amount of dye bound, and extend the pH range where appreciable binding occurs. This is also shown in Fig. 1. An increase in the concentration of buffer ions may be expected either to decrease or increase binding depending on the nature of the anions and cations used. As mentioned above, all ions may be expected to compete to some extent with dyes of like charge, but competition is usually greater with larger buffer molecules.[23] Also, as discussed by Danielli,[38] the pH near protein surfaces may be markedly different from that in the dye solution, and the difference is accentuated by increased ionic strength. The effect of salts on dye binding has also been discussed by Neale.[39]

3. Fixation

Several factors are obviously of importance in determining the effects of fixation on dye binding. Certain tissue constituents may be soluble in the fixative, and be removed, at least in part, by it. For example, some tissue proteins are apparently not immediately precipitated by 10% neutral formalin, and are removed from cells in smears, or from the outer portion of tissue blocks. Fixative may also alter electrostatic charges on nucleoprotein complexes by combining with ionizable groups. For example, formalin is known to combine with amino groups, and the metal cations in many fixatives, such as mercury and chromium, may be strongly bound by carboxyl and phosphoryl groups. Because the effects of complicated histological fixatives are obviously highly involved and often unpredictable, many workers have preferred to use simple fixatives, such as acetic alcohol or freeze-drying followed by alcohol treatment. Enzymes (deoxyribonuclease and ribonuclease) work well after such fixatives, and acid extraction of nucleic acids is also readily performed. Brachet[31] has found dye binding to be somewhat depressed after freeze-drying, however, possibly because of lipid interference.

4. Affinity

Methyl green shows, under certain special conditions, an affinity for DNA but not for PNA.[14,40,41] From the *in vitro* studies discussed above, it is evident that more methyl green is bound to highly polymerized than to depolymerized nucleic acid. The conditions under which methyl green stains DNA in tissue sections thus appear to be those in which the equilibrium factors are adjusted so that the affinity for the lower polymer PNA

[37] N. D. Levine, *Stain Technol.* **15**, 91 (1940).
[38] J. F. Danielli, *Biochem. J.* **35**, 470 (1941).
[39] S. M. Neale, *Trans. Faraday Soc.* **42**, 473 (1946).
[40] N. B. Kurnick, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 141 (1947).
[41] C. Leuchtenberger, *Chromosoma* **3**, 499 (1950).

is negligible, but that for DNA is strong enough to cause an appreciable binding. If the equilibrium is shifted toward an increased nucleic acid binding, however, the "specificity" breaks down, and both DNA and PNA stain. This shift may be brought about by blocking amino groups through strong formalin fixation,[42] by acetic anhydride, by removal of amino groups with nitrous acid,[33] or by increasing the pH of staining to 5.0 or above. It is obvious that methyl green may be used as a "specific" stain for DNA only where the factors determining the dye equilibrium are carefully controlled.

As shown by Unna,[43] treatment of slides for 10 minutes with water at 90° C. will abolish methyl green staining although the Feulgen reaction is still strong. Kurnick[14] postulated that the loss of staining was due to depolymerization of the DNA, and Pollister and Leuchtenberger[44] suggested that DNA stainability, when compared with the Feulgen reaction, might be an indicator of the degree of polymerization or at least of the steric condition of the DNA in tissues. This conclusion now seems unlikely for several reasons. (1) True depolymerization as caused by treatment with deoxyribonuclease results in removal of DNA from the slide. Appreciable DNA removal does not occur in the 15 minutes necessary to inhibit methyl green binding. (2) Heat depolymerization of DNA in solution as studied by Kurnick[17] took longer to complete, and then resulted in a decrease in dye binding of only 60 to 70 %. The loss of stain in tissue is complete with much shorter treatment. (3) The DNA stainability may be brought back after hot water treatment by blocking or removal of the protein amino groups.[33] None of these arguments completely disproves the possibility that some slight depolymerization or steric change may occur and that the reduced affinity of methyl green is an indication of it. A more plausible theory, however, is that the hot water possibly releases more amino groups to bind DNA phosphoryl groups, or at least to alter the charge in their vicinity, and thereby reduces the availability of phosphoryl groups to the dye. A slight reduction in azure B binding was reported by Alfert[33] after similar hot water treatment, and a slight decrease in toluidine blue binding *in vitro* was reported upon heat treatment of a PNA fibrinogen mixture by Herrman, Nicholas, and Boricious.[8] Although the variations in methyl green binding that have been reported in various tissues[41,45,46] are of interest, their interpretation at present is obscure, and may merely involve alterations in the amount or nature of adjacent proteins.

Although all basic dyes will show binding with nucleic acids in tissue

[42] H. Swift, *Physiol. Zool.* **23**, 169 (1950).
[43] P. G. Unna, *Monatsh. prakt. Dermatol.* **62**, (1887).
[44] A. W. Pollister and C. Leuchtenberger, *Proc. Natl. Acad. Sci. U.S.* **35**, 111 (1949).
[45] P. B. Weisz, *J. Morphol.* **87**, 275 (1950).
[46] N. J. Harrington and R. W. Koza, *Biol. Bull.* **101**, 138 (1951).

sections, it is obvious to anyone who has worked with them that they may differ rather markedly in the staining picture produced. For example, the extremely strong and partially nonspecific binding shown by crystal violet may be compared with the weak staining of the closely related methyl green. The individual behavior of dyes can serve to emphasize that binding is not a function of simple electrostatic forces alone. The binding strength, the number, and distribution of the auxochrome groups, and their dissociation characteristics, the ability of the dye to form molecular aggregates, and the effective volume of the dye molecule in penetrating crevices in the tissue—all of these factors are probably involved in reinforcing or counteracting electrostatic attraction.

5. Differentiation

The treatment of tissues after staining is often as important as the staining itself. When the slide is transferred to a rinse to remove the "unbound" dye, it is obviously subjected to a series of additional factors that markedly alter the original dye-nucleic acid equilibrium. The pH of the rinse is of great importance. If lower than the stain solution, in the case of a basic dye, it will reduce the binding affinity and result in loss of much dye previously bound. If higher, it may increase the dye bound since some imbibed dye may be held by the section before it is washed out. If the rinse has the same pH as the staining solution, the equilibrium is still drastically shifted toward dissociated dye, and the tissue will continue to lose stain at a rate determined partly by the dye-nucleic acid bond strength, dye solubility, temperature, and ion concentration.

Michaelis[47] suggested that alcohol rinses after staining would differentiate between bound and unbound dye. This obviously subjects the section to a new set of conditions, among which dye solubility seems to be particularly important. Certain dyes, e.g., methyl green and azure B, may be rapidly and completely removed by absolute ethanol so that the use of acetone or higher alcohols has been suggested; for example, *tert*-butyl alcohol.[41,48] Where the dye has a particularly low solubility in the rinse, this type of differentiation may not provide removal of all "unbound" dye. In most cases such as with methyl green-stained slides in *tert*-butyl alcohol, as in buffer solutions, the rinse continues to remove bound dye, so that it is necessary to set a purely arbitrary time limit for reproducible results.

If the staining picture desired is that at the pH and ionic strength of the stain solution, differentiation in solute minus stain for an arbitrary and controlled period of time seems desirable. Differentiation in nonaqueous media also gives good results, although such treatments may affect the

[47] L. Michaelis, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 143 (1947).
[48] M. H. Flax and M. Himes, *Physiol. Zool.* **25**, 297 (1952).

bond strength of the dye-nucleate, with consequent rapid or slow dye extraction.

One aspect of differentiation, much used by classical cytologists, is that of controlled "destaining." As mentioned below, more stain may be bound to structures with high nucleic acid concentrations, such as mitotic chromosomes and pycnotic or lymphocyte nuclei. These structures may have larger electrostatic fields, possibly because the ratio between available nucleic acid phosphoryl and protein basic groups is different than in the surrounding areas. In addition to binding more dye, such regions also apparently lose dye less rapidly, so that by using conditions causing heavy staining followed by controlled "destaining" as is done, for example, with iron hematoxylin, a point is reached where these structures alone are densely colored with a weakly staining background. By proper manipulation, "differential staining" of mitotic chromosomes is possible with a variety of basic dyes. In some cases factors other than electrostatic forces may also be involved, such as cell permeability to the dye-iodine complex in the Gram reaction.[49]

6. Metachromasy

Many basic dyes stain certain cell constituents one color and others another. This characteristic has been studied particularly by Lison[29] and Michaelis.[47,50] It deserves brief mention here because, as shown by Flax and Himes,[48] it affords a very simple way of differentiating PNA and DNA in tissues, and also may, when the phenomenon is better understood, provide some information on the arrangement of stainable groups in the nucleic acid molecule. Many basic dyes in solution do not show a linear relationship between concentration and optical density (Beer's law). With increasing concentration the color of the dye shifts and one or two new absorption peaks appear on the shorter wavelength side of the original absorption maximum. Several workers[47,51,52] have suggested that the three peaks represent dye monomers, dimers, and higher polymers, respectively, formed through Van der Waals interactions. In tissues, the polymer peaks are particularly characteristic of the sulfuric ester binding of mucin and chondroitin as pointed out by Lison.[29] Such substances stain red-purple with toluidine blue, while nucleic acids stain blue. Bank and Bungenberg de Jong[53] attributed the red staining to areas of higher charge density. Differences in the color of staining in some cases afford an excellent way to

[49] T. Mittwer, J. W. Bartholomew, and B. J. Kallman, *Stain Technol.* **25,** 169 (1950).
[50] L. Michaelis, *J. Phys. & Colloid Chem.* **54,** 1 (1950).
[51] E. Rabinowitch and L. Epstein, *J. Am. Chem. Soc.* **63,** 69 (1941).
[52] S. E. Sheppard, *Revs. Mod. Phys.* **14,** 303 (1942).
[53] O. Bank and H. G. Bungenberg de Jong, *Protoplasma* **32,** 489 (1939).

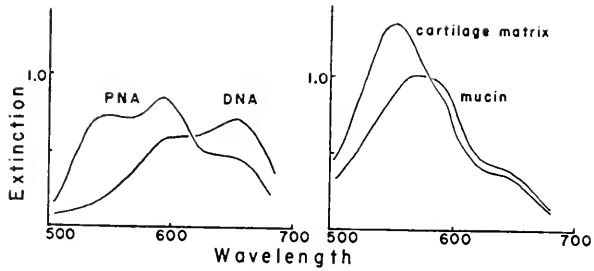


FIG. 3. Metachromasy of azure B in acetic alcohol-fixed salamander (*Ambystoma*) tissues. Absorption curves run with microphotometer on sections 8μ thick. DNA from nucleus after ribonuclease, showing predominant blue-green orthochromatic peak; PNA from Nissl substance, showing purple metachromatic peaks; mucin and cartilage matrix showing reddish PNA metachromatic peaks markedly different from PNA. Dye concentration 0.2 mg./ml. at pH 4.0; stained for 2 hours at 40°. [From Flax and Himes[48].]

distinguish basophilia of sulfuric esters from that of nucleic acid phosphoryl groups. Absorption curves for azure B, made on small regions of tissue with a microphotometer by Flax and Himes[48] are shown in Fig. 3. The curves for cartilage matrix and goblet cell mucin are markedly different than those of nucleic acids.

Under appropriate staining conditions nucleic acid may also show metachromatic staining.[53-55] Further, Flax and Himes[48] demonstrated that the conditions may be so adjusted that DNA stains mainly orthochromatically, and PNA metachromatically, so that DNA is colored a blue-green, cytoplasmic and nucleolar PNA a red-purple. Absorption curves for DNA and PNA staining are shown in Fig. 3.

In general, conditions which favor increased nucleic acid binding, such as increasing pH and dye concentration, or removal of amino groups with nitrous acid, favor increase in the dimer or polymer peaks. With a decrease in binding, brought out by lowering pH and concentration, and partially blocking phosphoryl groups with chromic acid, the monomer peak is more prominent. This would support the concept that dye interaction and consequent color change takes place after binding, and for structural reasons occurs more readily with PNA than DNA. It is also possible, though probably less likely, that dye intraction occurs before binding, and that PNA has a higher affinity for polymers and DNA for monomers. Flax and Himes[48] have also showed that, for one set of staining conditions, the shape of the absorption curve of azure B with PNA or DNA is approximately constant whether from regions of light or heavy dye binding. This would imply that polymerization occurs mainly between dye ions bound to the

[54] G. B. Wislocki, H. Bunting, and E. W. Dempsey, *Am. J. Anat.* **81**, 1 (1947).

[55] M. Weissman, W. H. Carnes, P. S. Rubin, and J. Fisher, *J. Am. Chem. Soc.* **74**, 1423 (1952).

same nucleic acid molecule, and less between those from adjacent nucleic acid molecules.

7. Quantitative Aspects

From the variables discussed above, we may conclude that dye binding in tissue sections, at least under the conditions generally used, cannot be trusted to give an accurate picture of the amount of nucleic acid present. In many cases, however, a rough indication of nucleic acid concentration may be all that is desired. In such cases basic dye binding will continue to be extremely useful, as it has been in the past. It is obvious from basic staining that more cytoplasmic PNA occurs in rat pancreas than liver, for example, or in root meristems than in differentiated regions. Also where protein-nucleic acid ratios are not markedly altered or the type of protein changed, estimates of the PNA concentration, made from photometric determinations of dye bound, may be reasonably accurate. A good correspondence between ultraviolet absorption and azure B binding was found in cytoplasm of mouse oocytes in various stages of growth[56] but a very poor correlation in cytoplasm of human liver.[57] Yolk platelets, at least in some cases, are only weakly basophilic, yet chemical determinations have shown them to be rich in PNA.[58]

In measurements on nuclei, basic dyes may show poor correlation with data obtained by ultraviolet absorption or the Feulgen reaction. As discussed later, the Feulgen reaction demonstrates a rather surprisingly regular stoichiometry with DNA. Basic dye binding, after ribonuclease, may be compared with the Feulgen reaction, run on adjacent sections. Methyl green and azure B gave values for small, dense erythroblast nuclei in newborn mouse liver about 50% higher than found for nuclei of liver parenchyma. The Feulgen reaction, however, gave no significant difference between the two types of nuclei. Similarly, about 35% more methyl green was bound to metaphase chromosomes from the small intestine than expected from Feulgen measurements. A better correlation between methyl green, Feulgen, and ultraviolet measurements was found by Frazer and Davidson[59] for rat liver and kidney, but widely different nucleic acid concentrations were probably not encountered. Preliminary photometric measurements with gallocyanine-chrome alum have shown a better correspondence to Feulgen staining than with other basic dyes;[60] its use for

[56] M. Flax, Ph.D. Thesis, Columbia University, New York, 1953.

[57] A. W. Pollister, J. Post, J. G. Benton, and R. Breakstone, *J. Natl. Cancer Inst.* **12**, 242 (1951).

[58] J. Panijel, "Le métabolisme de nucléoprotéines dans la gamétogenése et la fécondation." Hermann, Paris, 1951.

[59] S. C. Frazer and J. N. Davidson, *Exptl. Cell Research* **4**, 316 (1953).

[60] H. Swift, *Intern. Rev. Cytol.* **2**, 1 (1953).

quantitative estimates has been suggested,[61] although in some cases nonspecific staining may occur.[62] The binding of dyes mordanted with heavy metal ions to produce dye lakes is extremely strong and will take place at very low pH levels (e.g., 0.8 for gallocyanine).[61] Further investigation on the stoichiometry of such reactions would be of interest.

After treatment with deoxyribonuclease, the nuclei in many tissues do not stain at all with basic dyes, except for the nucleoli. Ultraviolet measurements on onion and lily nuclei, however,[60,63] indicate the presence of a considerable amount of material with a nucleic acid absorption curve that is removable with ribonuclease. It seems likely that this material represents PNA, where the phosphoryl groups are not available to basic dye binding, possibly because of protein blocking.

Because of the variables mentioned here, it seems that certain differences that have been reported in dye binding between tissues should be interpreted with some caution. Any "increase" in nucleic acid with mitosis needs to be investigated with other independent techniques such as ultraviolet absorption. Also the extent of "linkage" between, for example, DNA and histone probably cannot be estimated accurately by determining the increase in basic dye binding caused by histone removal. Histone removal would be expected to shift the equilibrium in the staining solution toward increased binding, but the increase produced would be of different magnitude depending on dye pH, concentration, etc. Kaufmann, McDonald, and Gay[64] reported that methyl green staining of onion root nuclei could be abolished, under certain conditions, either by deoxyribonuclease or ribonuclease. Rather than concluding that PNA is essential to DNA polymerization, a plausible explanation would seem to be that the amino groups known to be uncovered by PNA removal are sufficient in some cases successfully to inhibit methyl green binding by the DNA.

In conclusion it should be emphasized that, for a qualitative picture of nucleic acid distribution in tissues, dye binding may be a valuable research tool. If used properly basic dyes are practically specific for nucleic acid, and under special conditions can differentiate between DNA and PNA. The staining of tissues, at least as usually carried out, is subject to too many variables, however, to provide stoichiometric binding to nucleic acid, and thus dye content, as measured by photometric instruments, cannot be relied upon to give an accurate estimate of the amount of nucleic acid present. In certain cases measurements of dye bound may provide accurate

[61] L. Einarson, *Acta Pathol. Scand.* **28,** 82 (1951).
[62] U. Stenram, *Exptl. Cell Research* **4,** 383 (1953).
[63] H. Swift, *Texas Repts. Biol. and Med.* **11,** 755 (1953).
[64] B. P. Kaufmann, M. R. McDonald, and H. Gay, *J. Cellular Comp. Physiol.* **38,** Suppl. 1, 71 (1951).

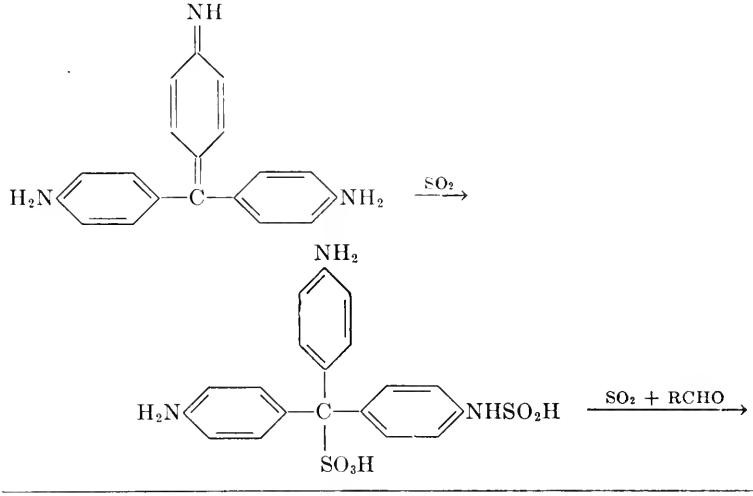
information, but in other cases they may not. For this reason results obtained from measurements of dye intensity should be interpreted with caution, and wherever possible checked by independent methods.

III. The Feulgen Reaction

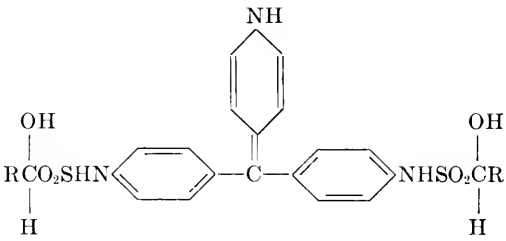
In spite of the tremendous number of studies on the Feulgen reaction, many points concerning it are still obscure. A few years after the published description by Feulgen and Rossenbeck[65] in 1924, it was employed by a great many cytologists for chromosome studies, where the interference of cytoplasmic staining could be eliminated, and chromosomal material could be distinguished from nucleoli. In the last few years the Feulgen raection has also been widely used, in conjunction with microphotometric instruments, to estimate the amounts of DNA in nuclei. To evaluate such studies, variables affecting Feulgen intensity should be examined in some detail. For a recent review, see Lessler.[66]

1. Chemistry

The mechanism of the Schiff reaction with aldehydes, which forms the basis for the Feulgen reaction, was studied by Wieland and Scheuing.[67] According to the theory proposed, the Schiff reagent, formed by the reaction of SO_2 with an acidic solution of basic fuchsin, produces a colorless compound, the N-sulfinic acid of *p*-fuchsinleucosulfonic acid, with the formula shown below:



[65] R. Feulgen and H. Rossenbeck, *Z. physiol. Chem.* **135**, 203 (1924).
[66] M. A. Lessler, *Intern. Rev. Cytol.* **2**, (1953).
[67] H. Wieland and G. Scheuing, *Ber.* **B54**, 2527 (1921).



This must combine with two aldehyde groups to restore the quinoid structure associated with the colored compound. Postulated intermediate steps were (1) the coupling of one aldehyde group with the sulfinic group of fuchsin-sulfonic acid, (2) the change of another amino group into a sulfinic group, and (3) the coupling with a second aldehyde, and consequent release of the sulfonic acid group from the central carbon. The aldehyde-Schiff complex produced is obviously a different dye than the original basic fuchsin, as may be shown by absorption curves of tissues.[68] It is readily distinguished by its magenta, rather than red, coloration.

The Feulgen reaction typically shows two absorption maxima (Fig. 4) denoting the presence of two chromophores, and under different conditions of staining these two components may vary independently. Since acidic solutions of basic fuchsin are also capable of combining with aldehyde groups,[69,70] presumably through some type of amino-aldehyde linkage, it seems possible that recoloration may proceed through combination of the tissue aldehyde groups with either the amino or sulfinic groups of the leucofuchsin molecule.

As mentioned by Conn,[71] commercial samples of basic fuchsin show some variation, most being mixtures both of pararosaniline (triaminotriphenylmethane acetate) and its mono- or dimethyl derivatives. The compound nature of the preparations does not seem to interfere with their stainability, although for quantitative work it is probably best to obtain pararosaniline, in as pure form as possible. Preparation of the Schiff reagent has been carried out in many different ways.[72-74] Although the source of SO_2 does not seem of much consequence, the amount present in the reagent can affect its sensitivity (see below). The fuchsin-sulfonic acid, once formed,

[68] R. E. Stowell and V. M. Albers, *Stain Technol.* **18,** 57 (1943).
[69] E. D. DeLamater, *Stain Technol.* **23,** 161 (1948).
[70] G. Gomori, "Microscopic Histochemistry." Univ. of Chicago Press, Chicago, 1952.
[71] H. J. Conn, "Biological Stains," 6th ed. Biotech. Publications, Geneva, N. Y. 1953.
[72] W. B. Atkinson, *Stain Technol.* **27,** 153 (1952).
[73] R. L. Shreiner and R. C. Fuson, "The Systematic Identification of Organic Compounds." Wiley, New York, 1940.
[74] J. D. Barger and E. D. DeLamater, *Science* **108,** 121 (1948).

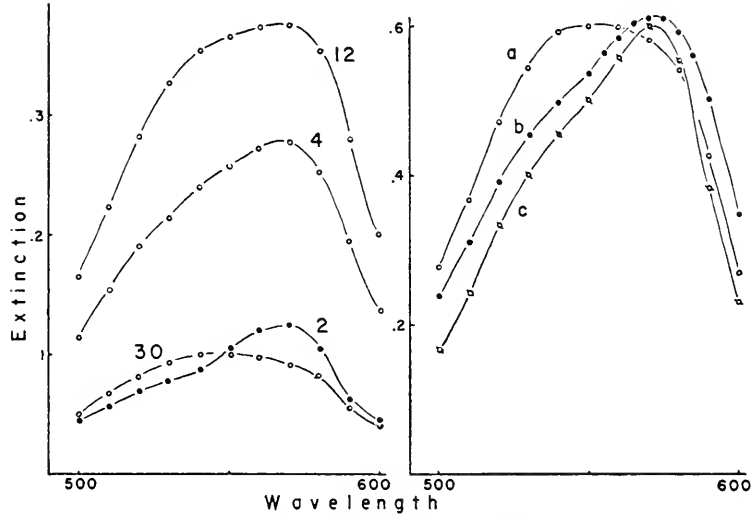


FIG. 4. Absorption curves of Feulgen dye. *Left:* Absorption curves made with microphotometer on single uncut mouse liver interphase nuclei (diameter about 8 μ) fixed in acetic alcohol, showing change in shape with different hydrolysis times in N HCl at 60°. *Right: b.* Mouse liver nucleus fixed in Sanfelice; 12 minutes hydrolysis. *c.* DNA solution (0.35 mg./ml. in 1-cm. cuvet) hydrolyzed 12 minutes. *a.* Same solution but adsorbed on 20-μ section of heat-denatured acetic alcohol-fixed egg albumen during 12-minute hydrolysis; the section then stained in Feulgen reagent and measured with a microphotometer.

readily loses SO_2 to reform basic fuchsin. For this reason the reagent should be kept tightly stoppered, and if it turns pink may be decolorized again on addition of more SO_2. Yellow or brown contaminating substances in the reagent may markedly inhibit staining of tissues and should be removed with activated charcoal.[75] On standing, a white precipitate forms in the Schiff reagent, and when this becomes pronounced optimal staining may no longer be obtained.

2. Specificity

The Feulgen reaction depends upon the production of aldehydes by a mild acid hydrolysis of the deoxypentose of DNA. Hydrolysis has been performed with hydrochloric, sulfuric, nitric, citric,[76] perchloric,[77] or phosphoric[78] acid. It is evident that this hydrolysis actually does form aldehyde

[75] L. C. Coleman, *Stain Technol.* **13,** 123 (1938).
[76] G. Widström, *Biochem. Z.* **199,** 298 (1928).
[77] H. S. DiStefano, *Stain Technol.* **27,** 171 (1952).
[78] S. A. Hashim, *Stain Technol.* **28,** 27 (1953).

groups, since aldehyde coupling reagents, such as bisulfite,[79] semicarbazide, phenylhydrazine, hydroxylamine,[80] and cyanide,[70] greatly reduce the intensity of the reaction provided they are used after hydrolysis. Agents which esterify the aldehyde or change it to an alcohol also block the reaction.[81] Also some other reactions for aldehydes give a positive test for nuclei after hydrolysis, for example, silver ammoniac solutions[82] and hydrazine.[83,84] Other aldehyde tests may fail to react.[81]

Why aldehyde groups should be produced by DNA and not PNA is not altogether clear. It has been pointed out by several workers[85] that acid hydrolysis removes most PNA from tissues. The specificity cannot rest on this alone, however, as intimated by Carr,[86] since PNA *in vitro* is also completely negative. Feulgen and Rossenbeck[65] demonstrated that the purine-sugar bond of DNA was split in hydrolysis to liberate the aldehyde, and suggested that the PNA purines were not so readily liberated. Overend and Stacey[87] synthesized a number of normal and deoxysugars in both pyranose and furanose forms. They reported that some sugars may occur naturally in a noncyclic aldehydo form and that this was present in significantly greater amounts in deoxyfuranose sugars than in those with a deoxypyranose or normal furanose structure. According to this view, the hydrolysis makes the aldehyde groups available both through rupture of aldehydo bonds involved in polymerization, and also by the liberation of purines.

A properly performed Feulgen reaction is practically specific for DNA, as has been demonstrated by many workers. DNA removal, through the use of purified pancreatic deoxyribonuclease prepared according to McCarty,[88] will render a tissue Feulgen-negative, while prolonged trypsin digestion, though it may destroy the integrity of the tissue, has little effect on the Feulgen intensity of remaining tissue fragments.[89] A number of investigators have tested Feulgen specificity with drops of DNA- or PNA-protein mixtures on slides.[70,80] These tests also demonstrate that proteins, and PNA from yeast, liver, or tobacco mosaic virus are completely Feulgen-negative.

There are a few other substances occurring in tissues that are likely to

[79] J. Brachet, *Experientia* **2**, 142 (1946).
[80] M. A. Lessler, *Arch. Biochem. and Biophys.* **32**, 42 (1951).
[81] J. F. Lhotka and H. A. Davenport, *Stain Technol.* **26**, 35 (1951).
[82] R. Feulgen and K. Voit, *Z. physiol. Chem.* **137**, 272 (1924).
[83] E. Herman and E. W. Dempsey, *Stain Technol.* **26**, 185 (1951).
[84] A. G. E. Pearse, *J. Clin. Pathol.* **4**, 1 (1951).
[85] J. O. Ely and M. H. Ross, *Anat. Record* **104**, 103 (1949).
[86] J. G. Carr, *Nature* **156**, 143 (1945).
[87] W. G. Overend and M. Stacey, *Nature* **163**, 538 (1949).
[88] M. McCarty, *Bacteriol. Revs.* **10**, 63 (1946).
[89] D. G. Catcheside and B. E. Holmes, *Symposia Soc. Exptl. Biol.* **1**, 225 (1947).

give a positive Feulgen reaction. Naturally ocurring aldehydes produce a pink color without acid hydrolysis. These include lipids, which may remain in tissues after formalin or osmic acid fixation, constituents in elastic fibers, and certain materials in plant cell walls, particularly those of xylem elements.[90] In inadequately washed tissues formaldehyde or paraldehydes may remain, to give a positive reaction. Some fixatives, particularly those containing chromic acid or other oxidizing agents, partially oxidize glycogen, mucin, starch, cellulose, and other polysaccharides, so that these give a pink color with Schiff's reagent. Also where sections are inadequately rinsed in the SO_2-HCl baths after staining, they may turn a more or less uniform pink in water or alcohol. All of these false reactions are readily distinguished from DNA staining since they produce a colored compound without previous hydrolysis, and their extent can be estimated by comparing hydrolyzed slides with unhydrolyzed controls. In some cases materials that produce a pink color in unhydrolyzed control sections are removed by the Feulgen hydrolysis. Since the Schiff reagent is acid, it slowly hydrolyzes DNA, so that previously unhydrolyzed tissues may be stained if allowed to remain in the reagent for long periods (6 hours or more).

Because interfering substances are quite common, for all new tissues to be investigated unhydrolyzed controls should be run, particularly if quantitative techniques are to be used. Feulgen-positive material should also be investigated with acid or enzyme extraction techniques where its DNA nature is doubtful. Such investigations would clarify, for example, the nature of the cytoplasmic granules in brain tissue reported by Liang[91] and Chu.[92] Also, one should never conclude on the basis of a negative Feulgen reaction, that DNA is "absent" from a nucleus. Even where all steps have been properly followed, no perceptible color can be seen in some nuclei, for example, in many mature oocytes. The DNA in such nuclei is obviously too dilute to produce a visible reaction.

3. Localization

A few investigators, particularly Stedman and Stedman,[93-95] have suggested that DNA is made readily diffusible by hydrolysis, and may produce, on contact with the fuchsin-sulfurous acid, a soluble dye that is adsorbed by various cell structures. The localization of dye given by the Feulgen reaction is thus thought to have little or no relation to the actual distribution of DNA. A great many people have argued against this sug-

[90] B. B. Hillary, *Botan. Gaz.* **101**, 276 (1939).
[91] H. M. Liang, *Anat. Record* **95**, 511 (1947).
[92] C. H. Chu, *Science* **106**, 70 (1947).
[93] E. Stedman and Ellen Stedman, *Nature* **152**, 267, 503 (1943).
[94] E. Stedman and Ellen Stedman, *Symposia Soc. Exptl. Biol.* **1**, 232 (1947).
[95] E. Stedman and Ellen Stedman, *Biochem. J.* **47**, 508 (1950).

gestion.[70,96-99] Although the possibility that some slight alteration in DNA localization may take place during hydrolysis is difficult to exclude entirely, and definitely may occur with over-hydrolysis, this is very obviously not an important factor in the usual Feulgen method. Dye distribution is essentially unchanged whether the Feulgen reaction is carried out on whole tissue blocks,[100] on very thin smears, or on cell homogenates. It is similar in very early and mid-stages of hydrolysis. Prolonged washing after hydrolysis has little effect. Moreover, the distribution of stain, caused by adding excess DNA to the hydrolysis bath, or hydrolyzed DNA to the staining solution, where adsorption actually does occur, is completely different, showing nucleolar and cytoplasmic coloring.

4. Fixation

The type of fixation used is important in determining the maximum intensity obtainable. It also causes variations in the effects of hydrolysis, and even in the color of the Feulgen complex itself (Fig. 4). Nuclei from tissues fixed in neutral formalin are usually more intense than after acetic alcohol. In addition, when a block of tissue is fixed, the peripheral nuclei, with either acetic alcohol or formalin, are significantly darker than those toward the center of the block, as demonstrated by photometric determinations of the amount of dye per nucleus.[60] This variation in intensity may be as great as 30%. No such gradient is shown with fresh-frozen or frozen-dried material. When a fresh piece of tissue is placed in fixative, the composition of the fixing fluid obviously varies as it penetrates the block. Dilution of fixative occurs, and certain constituents penetrate more rapidly than others. Also certain constituents soluble in the fixative are more readily removed from the outer cells. In Table I are shown measurements on nuclei from the same rat liver, fixed in the same fixative, and stained together. A 25% difference was found between inner and outer nuclei. When cells were homogenized in sucrose before fixation, centrifuged down, and resuspended in fixative, the Feulgen values were still higher. From an analysis of the absorption curves of these nuclei, one can conclude that the difference is not caused by variation in dye distribution, but rather by actual differences in the amounts of dye bound per nucleus. From measurements on nuclei stained with the Millon reaction for tyrosine,[101] some protein, possibly the globulin fraction,[102] appears to be partially extracted

[96] R. E. Stowell, *Stain Technol.* **20,** 45 (1945).
[97] R. E. Stowell, *Stain Technol.* **21,** 137 (1946).
[98] J. Brachet, *Symposia Soc. Exptl. Biol.* **1,** 207 (1947).
[99] C. H. Li and M. Stacey, *Nature* **163,** 538 (1949).
[100] J. F. Lhotka and H. A. Davenport, *Stain Technol.* **22,** 139 (1947).
[101] E. M. Rasch and H. Swift, *J. Histochem. and Cytochem.* **1,** 392 (1953).
[102] W. R. Kirkham, *Federation Proc.* **11,** 240 (1952).

TABLE I

THE EFFECT OF FIXATIVE PENETRATION ON FEULGEN INTENSITY[a]

	Feulgen dye bound	Standard error
Liver (Tetraploid nuclei)		
Center of section	6.4	0.05
Edge of section	7.9	0.08
Sucrose homogenate	9.0	0.16
Frozen section	8.8	0.08
Spermatocytes		
Center of section	8.2	0.08
Edge of section	9.1	0.07
Sucrose homogenate	9.3	0.16

[a] Values in arbitrary units represent means of 15 measurements on formalin-fixed rat nuclei. All tissues were from the same animal.

from the nuclei with higher Feulgen values. It is possible, then, that this fraction, where present, may partially inhibit the Feulgen recation. Lhotka and Davenport[81] found a more intense reaction if tissues were kept 5 minutes between removal and fixation in picrosulfosalicylic acid. Protein loss, through autolysis, may be responsible for the difference. The increased Feulgen intensity found in early pycnosis of tumor nuclei[42] is possibly also due to protein loss.

Certain tissues are more readily penetrated by fixatives than others. For example, the structure of rat testis fragments enables a more rapid fixative penetration than denser tissue such as liver. Thus under some conditions, where amounts of dye per nucleus are to be compared between two tissues, the error due to penetration may cause misleading results. Fixation is naturally more uniform with adequately frozen-dried or homogenized tissues, and such treatment may occasionally be necessary where accurate comparisons are wanted.

The effects of cytological fixatives on Feulgen-stained slides have been discussed by several investigators.[90,103,104] Most fixatives give adequate results where fixation has not been prolonged, where tissues have been adequately washed, and where strong oxidizing agents, such as 5% chromic acid, have been avoided.

5. HYDROLYSIS

Many workers have described the effects of hydrolysis time on Feulgen intensity.[85,90,103,105-107] Typical curves are given in Fig. 5 for hydrolysis in

[103] H. Bauer, *Z. Zellforsch.* **15**, 225 (1932).
[104] P. F. Molovidov, *Protoplasma* **25**, 570 (1936).
[105] T. Caspersson, *Biochem. Z.* **253**, 97 (1932).

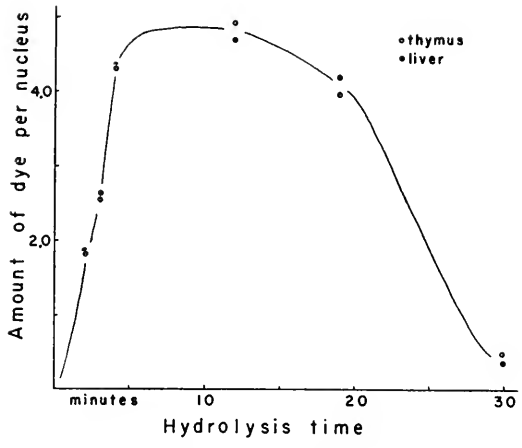


FIG. 5. Effect of hydrolysis time on the amount of Feulgen dye bound per nucleus. Tissues fixed in acetic alcohol and sectioned at 20 μ. Each point represents the mean of 12 tetraploid class interphase nuclei from mouse liver and thymus, measured at 560 mμ. Dye expressed in arbitrary units. Density readings at 30 minutes are too low to be accurate, but are below 0.6.

N HCl at 60° C. As hydrolysis time is increased, the intensity increases to a maximum (about 12 minutes for acetic alcohol-fixed mouse tissues) and then declines. The increase represents formation of aldehydes, and is associated with removal of purines from the tissue.[85] The subsequent decrease involves actual loss of DNA from the tissues and possibly also chemical breakdown. Ely and Ross[85] reported that material capable of giving a positive Feulgen reaction was extracted from a suspension of isolated thymus nuclei. The actual extent of DNA removal was not estimated. When fixatives that contain chromic acid or bichromate are used (e.g., Flemming's, Navashin's, Sanfelice) the intensity reaches its maximum somewhat later, and a long plateau occurs before DNA is lost. Measurements of Flemming-fixed frog cartilage nuclei showed maximum intensity from 20 to 50 minutes in *N* HCl at 60° C.[107] Such fixation, if prolonged, enables hydrolysis to proceed to a maximum before DNA removal, which must be inhibited through interaction with the heavy metal. The intensity obtained with chromic acid fixatives in general is no greater than with acetic alcohol, however, so that one might conclude that fixation in fluids without chromic acid enables hydrolysis to be near maximal before appreciable DNA is removed.

Maximum hydrolysis time may be different for different species. For example, Hillary[90] found hydrolysis in the alga *Spirogyra* needed to be

[106] E. D. DeLamater, H. Mescon, and J. D. Barger, *J. Invest. Dermatol.* **14**, 133 (1950).
[107] H. S. DiStefano, *Chromosoma* **3**, 282 (1948).

longer than for most plants (15 to 20 minutes), and McMaster[108] found acetic alcohol-fixed sea urchin eggs stained maximally after only 8 minutes, instead of the usual 12 minutes for most vertebrate nuclei. Where Feulgen intensity is to be compared between species, these differences may be of considerable importance. A difference in optimal hydrolysis time for different tissues of the same animal has also been described, although Feulgen intensity was determined only by eye.[106] When such tissues are measured by a microphotometer, and the amount of dye per nucleus computed, there is not significant difference between the hydrolysis curves (Fig. 5). Thus, although hydrolysis maxima may differ between species, there appears to be almost no evidence of a difference between tissues of the same species. Sperm nuclei, however, are an exception. Formalin-fixed mouse sperm nuclei start to degenerate after about 20 minutes of hydrolysis, although other nuclei, including the spermatocytes, remain intact. In the annelid *Sabellaria*, acetic alcohol-fixed sperm show appreciable disintegration even after 12 minutes,[109] although other testis nuclei do not. This change is probably influenced by the alteration during sperm formation in basic protein to which the DNA is bound. One other exception studied by Hillary[90] is found in plant nuclei with high tannin content. Hydrolysis time curves, even in acetic alcohol-fixed tissues, are altered to the chromic acid type, so that such nuclei would not be optimally hydrolyzed at 12 minutes, and thus would be paler than in surrounding cells. Such nuclei are often noticeably brownish, for example, in fern epidermis. In addition, Patau and Srinivasachar[110] have reported rather unpredictable changes in amounts of dye bound by mitotic stages in onion, after various hydrolysis or posthydrolysis treatments; for example, certain treatments were found to affect prophase nuclei more than metaphases, and others had the opposite effect.

The shape of the Feulgen dye absorption curve shifts during hydrolysis, as shown in Fig. 4. The reason for this is not altogether clear. In general, nuclei on one slide show curves all roughly of one shape, regardless of tissue, but in some cases differences in distributions of the two components within nuclei have been found.[111] Such variations in the color produced by the Feulgen reaction obviously should be considered where quantitative determinations are made, particularly where the two-wavelength method of measuring is employed.

6. Variations in the Feulgen Reagent

Preparations of fuchsin-sulfonic acid recommended by different authors show wide variation in dye, pH, and SO_2 content. The effect of these vari-

[108] R. McMaster, Ph.D. Thesis, Columbia University, New York, 1953.
[109] M. Alfert and H. Swift, *Exptl. Cell Research* **5**, 455 (1953).
[110] D. Srinivasachar, Ph.D. Thesis, University of Wisconsin, 1953.
[111] L. Ornstein, *J. Lab. Invest.* **1**, 85 (in discussion of paper by J. F. Scott) (1952).

ables has recently been studied by several workers.[72,85,112] Although constituents may be varied over rather wide limits and still produce a serviceable Feulgen reaction, for quantitative studies some control over the composition of the reagent is desirable. Most quantitative determinations on the sensitivity of the reagent have been made on formaldehyde or DNA solutions. The situation for nuclei may not be strictly comparable. More measurements on tissue sections after various treatments would be helpful.

As one would expect if recolorization proceeds according to Wieland and Scheuing,[67] excess SO_2 is needed in the reagent solution. With formalin solutions, Atkinson[72] found a concentration of 10 moles of bisulfite to 1 of dye was optimal, with a decrease in intensity with smaller or larger bisulfite concentrations. For DNA solutions, Ely and Ross[85] obtained an increasing intensity with metabisulfite concentration, and no optimal concentration was reached even when molar ratios were very much higher. This is an important variable, since the SO_2 content of the reagent readily changes through evaporation (Table II). The pH of the reagent solution also affects the intensity of the reaction, as shown in Table II. A reduction in color with decreasing pH has been described for formaldehyde[112] and DNA[85] solutions.

7. Quantitative Aspects

The relation between amounts of DNA and regenerated dye intensity *in vitro* was studied by Caspersson,[105] who concluded that estimates could be made to 2% provided proteins were not present in appreciable amounts. Rumpf[113] concluded that the Schiff reagent was not quantitative for aldehydes, although Atkinson[72] found a linear relation for formaldehyde. Lessler[80] used drops of DNA-gelatin mixtures on slides to test the linearity of color intensity, and found it was proportional to the DNA concentration only up to 1 mg. of DNA per milliliter of 20% gelatin. Above that concentration the intensity was depressed. The presence of histone under some conditions was found to enhance and under other conditions to depress the dye intensity.[114,115] Also the intensity decreases in the SO_2-HCl rinse solutions[110] at a rate dependent on their composition.[116]

In a few cases the amounts of DNA per nucleus, as determined biochemically, have been compared with the intensity of Feulgen-stained nuclei as measured with a microphotometer. The determinations made by Ris and Mirsky[117] with the two methods matched to within 10%. In each

[112] J. B. Longley, *Stain Technol.* **27**, 161 (1952).
[113] P. Rumpf, *Ann. chim.* (*Paris*) [11]**3**, 327 (1935).
[114] A. Shibatani, *Nature* **166**, 355 (1950).
[115] H. Naora, H. Matsuda, M. Fukuda, and A. Sibatani, *J. Japan. Chem.* **5**, 729 (1951).
[116] A. Shibatani, *Experientia* **8**, 268, 1952.
[117] H. Ris and A. E. Mirsky, *J. Gen. Physiol.* **33**, 125 (1949).

TABLE II

THE EFFECT OF VARIOUS TREATMENTS ON FEULGEN INTENSITY[a]

		Feulgen dye bound	Standard error
Composition of the Feulgen reagent			
pH ($Na_2S_2O_5$ concentration 1%)			
0.8		4.8	0.10
1.6		6.3	0.11
2.1		6.5	0.08
2.4		6.9	0.12
2.9		7.8	0.17
3.6		8.2	0.11
$Na_2S_2O_5$ concentration, % (pH 2.1)			
0.3		7.8	0.13
0.5		7.8	0.13
1.0		6.5	0.08
2.0		6.3	0.11
5.0		5.5	0.13
10.0		4.8	0.16
Time in the Feulgen reagent			
5 min.		5.3	0.11
15 min.		6.1	0.14
30 min.		6.1	0.12
1 hr.		6.6	0.11
24 hr.		6.8	0.15
48 hr.		5.6	0.11
Composition of rinses			
$Na_2S_2O_5$ conc., %	pH		
0.5	1.8	6.6	0.13
0.5	2.3	7.5	0.08
0.5	7.0	7.2	0.10
1.5	1.8	7.1	0.07
1.5	2.3	7.9	0.10

[a] Values in arbitrary units represent means of 10 measurements on tetraploid nuclei of formalin-fixed mouse liver. The Feulgen reagent, unless otherwise specified, contained 0.5% basic fuchsin (National Aniline, dye content 97%), 1% $Na_2S_2O_5$, and had a pH of 2.1.

case one tissue of known DNA content was used for reference. More extensive comparisons of this type are badly needed.

Certainly the most striking indication of the quantitative nature of the Feulgen reaction can be seen by an analysis of the data obtained when nuclei are measured. As indicated by Ris and Mirsky[117] and many others since then, the means of values for the polyploid classes in rat or mouse liver fall into a 1:2:4:8 series. With accurate measuring of normal adult liver, there is no overlap at all between classes, and the means match the theoretical geometrical progression by less than 5%. When liver is com-

pared with other tissues in the same organism, the lowest class is also present, and in some cases, such as pancreas and thymus, polyploid classes are also found. The means of these classes, from different tissues, also match each other closely even when the volumes of the different nuclei, and thus the DNA concentrations, vary widely. Certainly the most plausible explanation of such data is that there is a constant relation between the diploid chromosome set and the DNA content, so that each diploid nucleus of a species contains a characteristic amount of DNA, and also that the Feulgen intensity gives an accurate indication of the amount of DNA present. This question is discussed further in Chapter 19.

It is rather surprising that this relationship holds throughout the concentration range encountered in mouse nuclei. In Fig. 6, a linear relationship may be seen to hold even for concentrations of 0.3 g./ml. of DNA, about 300 times higher than the optimal concentration found by Lessler[80] for gelatin-DNA drops. The protein-DNA ratios must vary rather widely in these nuclei. Evidence for protein interference as described from *in vitro* studies,[105,114,115] however, is apparently absent, except possibly in the case of the fixation and pycnosis effects discussed above.

From these data, and also from data of several other laboratories (see Swift[60] for review) one may conclude that the Feulgen reaction can give quantitative information of considerable accuracy. This does not mean it should be accepted indiscriminately as a quantitative method. Where distributions of the type shown in Fig. 6 are obtained, one may conclude with a high degree of probability that the Feulgen reaction is quantitative for such material. If such a relation is not obtained, one will have to determine whether the Feulgen reaction or the photometric technique is at fault, or a real DNA variation is involved. Many biochemical color reactions are subject to variations when applied to different material, and the Feulgen reaction is probably no exception. Anyone using the Feulgen reaction as a quantitative method should obviously watch for variables in technique that may affect some nuclei differently from others.

In many of the quantitative studies that have been made with the Feulgen reaction, values have been expressed in arbitrary photometric units, and not in absolute amounts of DNA. Where absolute amounts are desired, in view of the many variables that cause slight alterations in intensity, the simplest procedure is to mount the unknown beside a control tissue with nuclei of known DNA content, such as rat or beef liver, and compare measurements from the two sections. Fixation and handling of the material should be identical with the control. Even so, variation may occur with fixative penetration unless freezing-drying or homogenates are used. Widely different organisms may also show different hydrolysis optima, and so

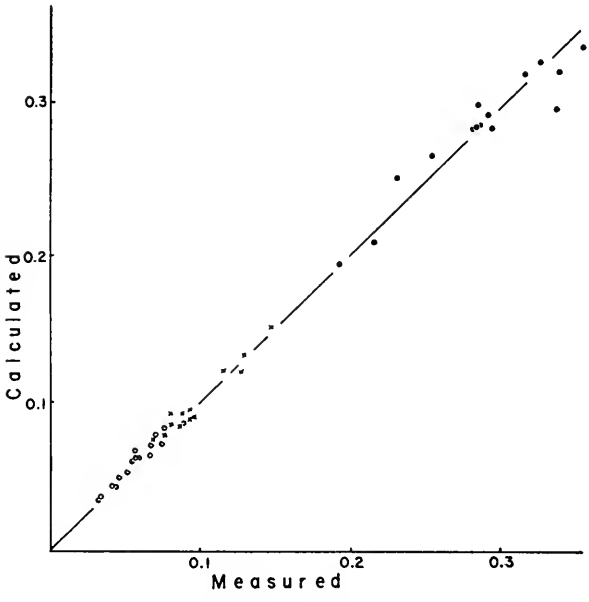


FIG. 6. Concentration of DNA in individual mouse nuclei, as measured with the Feulgen reaction and as calculated assuming a constant amount of DNA per nucleus. *Circles to left*, liver parenchymal nuclei; *crosses*, thymus nuclei; *dots*, normoblast nuclei from newborn liver. For each nucleus the dye concentration was determined; the expected dye content was calculated from the measured nuclear diameter, and a "standard" amount of dye assumed to be present in each nucleus. This standard was taken from the mean of 10 additional diploid adult liver nuclei from a section on the same slide. Ordinate and abcissa are in units of DNA concentration, 10^{-9} mg./μ^3, or g./ml., assuming each diploid nucleus contains 6×10^{-9} mg. DNA.

cannot be adequately compared. Good estimates of the amounts of DNA in nuclei were obtained, however, when nuclei from shad and chicken liver were compared with beef,[117] so that hydrolysis times are apparently similar for these vertebrates.

There have been a few reports that the Feulgen reaction may be used in conjunction with modifications in technique to distinguish between different types of deoxyribonucleoprotein. Sharma[118] reported that the heterochromatic segments of onion and bean chromosomes could be selectively stained by treatment with 0.25 *M* trichloroacetic acid at 60° before hydrolysis in HCl. Lessler[119] found that Feulgen blocking agents acted differently on erythrocytes of several different vertebrates.

[118] A. K. Sharma, *Nature* **167**, 441 (1951).
[119] M. A. Lessler, *J. Natl. Cancer Inst.* **12**, 237 (1951).

IV. Other Cytochemical Reactions

In addition to the Feulgen reaction, one other compound, which condenses with the sugar of nucleic acid, has been used as a cytochemical technique. This is 9-phenyl-2,3,7-trihydroxy-6-fluorone (or -xanthone), employed by Turchini and collaborators[120-122] and also by Backler and Alexander.[123] The compound when combined with PNA is yellow to pink and with DNA, blue to purple. It also combines with other sugars to give different shades. Turchini employed fixatives containing dichromate so that PNA will remain in the section during the partial hydrolysis. Backler and Alexander obtained good results with Zenker's, Bouin's, formalin, and frozen-dried sections, that compared favorably with gallocyanine-chrome-alum staining.

A method for localizing nucleic acid purines and pyrimidines through azo coupling has been proposed by Mitchell[124] and Danielli.[125-127] In this reaction coupling with tyrosine, tryptophan, and histidine is blocked by benzoylation, leaving purines and pyrimidines to react with tetrazotized benzidine. This compound, after coupling, can then be made an intense purple by further coupling with naphthol. The specificity of the reaction apparently has not been tested with nucleases. Gomori[70] has strongly criticized this method, stating that no coupling to bases can be expected to take place at all under the conditions suggested, and that diazonium decomposition products may be strongly and nonspecifically adsorbed to tissues. Clearly more study of this method is needed before it can be used with any confidence.

V. Extraction Techniques

In many cytochemical studies it is desirable to remove one or both nucleic acids from tissue sections. Ultraviolet absorption does not distinguish between the types of nucleic acids. If it is to be made specific for DNA or PNA in nuclei, these must be selectively removed. Also, evidence for the specificity of cytochemical reactions is obviously gained if "blank" slides from which the component studied has been extracted can be used for comparison.

[120] J. Turchini, P. Castel, and K. Kien, *Trav. soc. chim. biol.* **35,** 1329 (1943).
[121] J. Turchini, P. Castel, and K. Kien, *Bull. histol. appl.* **21,** 124 (1944).
[122] J. Turchini, *Exptl. Cell Research Suppl.* **1,** 105 (1949).
[123] B. S. Backler and W. F. Alexander, *Stain Technol* **27,** 147 (1952).
[124] J. S. Mitchell, *Brit. J. Exptl. Pathol.* **23,** 296 (1942).
[125] J. F. Danielli, *Symposia Soc. Exptl. Biol.* **1,** 101 (1947).
[126] J. F. Danielli, *Cold Spring Harbor Symposia Quant. Biol.* **14,** 32 (1951).
[127] J. F. Danielli, "Cytochemistry, A Critical Approach." Wiley, New York, 1953.

1. Acid Extraction

The technique of nucleic acid extraction with trichloroacetic acid, suggested by Schneider,[128] has often been performed on tissue sections. Complete extraction of nucleic acid has been reported after 5 or 10% TCA at 90° for 15 minutes.[129,130] Tests for tyrosine and arginine were unaffected.[130] Nucleic acids are usually readily removed from tissues fixed in acetic alcohol or neutral formalin. With fixatives containing chromic acid, some basophilia may remain even after treatment for 1 hour. Some tissues are extracted more readily than others, and the effectiveness of the treatment should always be tested with basic dyes or ultraviolet absorption. If the Feulgen reaction is used to check for DNA removal, it should be remembered that the HCl hydrolysis may further extract DNA remaining after the trichloroacetic acid treatment. In some cases nuclei are seen to be Feulgen-positive where the HCl hydrolysis is omitted, meaning that extraction may be incomplete, and that some trichloroacetic-hydrolyzed DNA remains.

Trichloroacetic acid extraction should be used with caution in connection with ultraviolet absorption measurements. Its use may markedly increase the refractive index of proteins in tissue sections and thus increase the nonspecific light loss. It may also alter the shape of the protein absorption curve in the region of nucleic acid absorption, due to oxidation of phenolic groups.

The use of perchloric acid for extraction of PNA from tissue homogenates was described by Ogur and Rosen.[131] It has been used on tissue sections by several investigators. In Zenker-fixed cat and rat tissues, 10% perchloric acid at 5° for 18 hours removed PNA selectively, provided frozen sections were used; paraffin-embedded material was less satisfactory.[132] With acetic alcohol-fixed rat pancreas, 6 minutes in 10% perchloric acid at 25° removed PNA from the cytoplasm, and also a 3-minute treatment removed about 40% of the nucleic acid from the nucleus as determined by ultraviolet absorption.[133] Presumably this also represented PNA, since the Feulgen reaction was undiminished until 9 minutes of treatment, after which time presumably the DNA was extracted. Apparently in this material PNA may be selectively removed, leaving the DNA in place, but time limits are

[128] W. C. Schneider, *J. Biol. Chem.* **161,** 293 (1945).
[129] A. W. Pollister and H. Ris, *Cold Spring Harbor Symposia Quant. Biol.* **10,** 147 (1947).
[130] J. Brachet and J. R. Shaver, *Stain Technol.* **23,** 177 (1948).
[131] M. Ogur and G. Rosen, *Arch. Biochem.* **25,** 262 (1949).
[132] N. M. Sulkin and A. Kuntz, *Proc. Soc. Exptl. Biol. Med.* **73,** 413 (1950).
[133] H. S. DiStefano, *Science* **115,** 316 (1952).

narrow. Similar results for protozoan cells have been reported by Seschachar and Flick.[134]

The studies of Koenig and Stahlecker[135] indicate that tissues may vary rather widely as to the ease of PNA extraction. Cat tissues fixed in 10% formalin were used. At 23° PNA was removed in 1 hour from liver, but only after 6 hours from nerve cells. At 4° PNA was not completely removed from nerve tissue, even after 96 hours. Prolonged exposure to formalin made the extraction very much more difficult.

The extent of the perchloric acid treatment needed for PNA extraction thus varies widely with different tissues and fixatives, and the proper conditions need to be determined in each case. Under some conditions very little time may elapse between PNA removal and reduction in ultraviolet absorption due to the removal of the DNA-purines. In addition, Pearse[136] reports that perchloric acid also removes various protein, lipoprotein, and glycoprotein fractions.

2. Nuclease Extraction

Enzymes are discussed here only in respect to their use in removing nucleic acids from tissue sections. They have already been dealt with in detail in Chapter 15. A number of different nuclease preparations have been used,[3,89,137,138] although those from beef pancreas have been most studied. Ribonuclease has been prepared by the method of Kunitz[139] with modifications suggested by McDonald.[140] Such preparations contain no measurable amounts of proteolytic activity either *in vitro* or on slides,[64,140,141] and, at least in many cases, show no measurable activity on DNA, as determined *in vitro*[142] or on slides with the Feulgen reaction.[64] The partial removal of DNA from liver nuclei by high ribonuclease concentrations has been described[143,144] but is possibly ascribable to deoxyribonuclease contamination. Contaminants may be present in commercial samples of crystalline ribonuclease or can easily arise with bacterial con-

[134] B. R. Sesachar and E. W. Flick, *Science* **110,** 659 (1949).
[135] H. Koenig and E. Stahlecker, *J. Natl. Cancer Inst.* **12,** 237 (1951).
[136] A. G. E. Pearse, "Histochemistry, Theoretical and Applied," p. 120. Churchill, London, 1953.
[137] R. J. Dubos and R. H. S. Thompson, *J. Biol. Chem.* **124,** 501 (1938).
[138] D. Mazia and L. Jaeger, *Proc. Natl. Acad. Sci. U.S.* **25,** 456 (1939).
[139] M. Kunitz, *J. Gen. Physiol.* **24,** 15 (1940).
[140] M. R. McDonald, *J. Gen. Physiol.* **32,** 39 (1948).
[141] B. P. Kaufmann, M. R. McDonald, H. Gay, K. Wilson, R. Wyman, and N. Okuda, *Yearbook Carnegie Inst.* **46,** 136 (1948).
[142] L. M. Gilbert, W. G. Overend, and M. Webb, *Exptl. Cell Research* **2,** 138 (1951).
[143] A. W. Pollister, M. Flax, M. Himes, and C. Leuchtenberger, *J. Natl. Cancer Inst.* **10,** 1349 (1950).
[144] A. W. Pollister, *J. Cellular Comp. Physiol.* **38,** Suppl 1, 87 (1951).

tamination of the enzyme or buffer solution.[145] Consequently, solutions of enzyme should be freshly prepared before use on tissues, and proteolytic activity should be destroyed by heating (for example, in a boiling water bath for 5 minutes in 0.2 saturated ammonium sulfate[140]). Where preparations are to be used for quantitative ultraviolet studies, the effect of the enzyme may also be tested on control Feulgen slides.

The type of fixation used can greatly alter the effectiveness of ribonuclease treatment.[31,141,146,147] The enzyme usually works easily on acetic alcohol-fixed tissues, at concentrations of 0.01 %. Although it will work after certain chromic acid fixatives (Flemming's, Navashin's), higher concentrations or longer digestion periods must be used. After Bouin's or Benda's fixation, the enzyme may not extract any appreciable PNA.[141] Different tissues vary in the ease of PNA removal. In certain cases, for example, in formalin or acetic alcohol-fixed biopsies of human cirrhotic liver,[57] ribonuclease had no perceptible effect and also acted incompletely on sections of rat liver.[148] Kaufmann *et al.*[149] found that nuclear PNA is less easily digested in onion root sections than PNA of the cytoplasm. This difference was attributed to the fact, found *in vitro*, that DNA partially inhibits ribonuclease action on PNA.

Ribonuclease is effective on tissue sections over a wide pH range (about 5 to 8). Buffers may seriously inhibit activity, and also particularly at alkaline pH may remove PNA from control sections in the absence of enzyme.[140,146] In addition, when basic dyes are used, buffer ions may compete for binding sites, as mentioned above. For these reasons enzyme solutions are best adjusted with dilute sodium hydroxide to a slightly acid pH (about 6.5).[31,150] Since ribonuclease is heat-stable, it may be used at elevated temperatures. Most rapid extraction occurs between 40 and 60°, although the higher temperature in some cases may cause increased nonenzymic PNA loss.[31]

Preparation of pancreatic deoxyribonuclease may be made according to the method of Fischer[151] or of McCarty,[88] and can be further purified by repeated ammonium sulfate fractionation.[152] This purification can effectively eliminate ribonuclease contamination, but some proteolytic activity

[145] W. Jacobson and M. Webb, *Exptl. Cell Research* **3**, 163 (1952).
[146] R. E. Stowell and A. Zorzoli, *Stain Technol.* **22**, 51 (1947).
[147] R. Tulasne and R. Vendrely, *Nature* **160**, 225 (1947).
[148] K. K. Tsuboi and R. E. Stowell, *Biochem. et Biophys. Acta* **6**, 192 (1950).
[149] B. P. Kaufmann, M. R. McDonald, H. Gay, N. Okuda, J. M. Pennoyer, and S. Blowney, *Yearbook Carnegie Inst.* **47**, 144 (1948).
[150] B. P. Kaufmann, M. R. McDonald, and H. Gay, *Am. J. Botany* **38**, 268 (1951).
[151] F. G. Fischer, I. Böttger, and H. Lehmann-Echternacht, *Z. physiol. Chem.* **276**, 271 (1941).
[152] W. G. Overend and M. Webb, *J. Chem. Soc.* **1950**, 2746.

may remain. Because of this the use of 0.1 % gelatin to stabilize deoxyribonuclease solutions[83] or of 0.05 *M* cysteine or hydroxylamine to inhibit proteolytic contaminants[145] has been suggested. For quantitative use an assay of proteolytic activity, for example, on hemoglobin,[153] is advisable. The presence of magnesium ions is necessary for pancreatic deoxyribonuclease digestion of DNA solutions.[88] Several workers, however, have found that magnesium ions are not necessary for action of the enzymes on tissues[138,154] although others disagree.[130] Use of the enzyme at about pH 6 has been recommended[150] because of the buffer effects mentioned above. With acetic alcohol fixation, good results are usually obtained with 30- to 60-minute treatment in a 0.01 % solution, at 37° or room temperature. Deoxyribonuclease acts very slowly on formalin-fixed tissues, and is completely inactive on tissues treated with fixatives containing chromic acid. Vercauteren[19,155] found that certain enzyme preparations were inactive on sections in spite of strong *in vitro* activity.

Nucleases can be an extremely important cytochemical tool, both for testing the specificity of nucleic acid reactions and also for adding specificity to otherwise nonselective methods, such as ultraviolet absorption. It is apparent, however, that they are not infallible, and in any quantitative work adequate controls must be run. Control slides should be treated with solutions minus the enzyme. Where nucleases are used to fractionate nuclei for ultraviolet studies, the effect of enzymes should be checked with basic staining and the Feulgen reaction, and, as an additional check, fractionation may be carried out twice, with the enzymes used in different order.

VI. Photometric methods for cell studies

Since the pioneer work of Caspersson,[156] there has been a rapidly growing literature on the photometry of cells, including several recent reviews.[157-162] Numerous measuring instruments have been constructed.[156-174] Some have

[153] M. L. Anson, *J. Gen. Physiol.* **22,** 79 (1938).
[154] L. M. Gilbert, W. G. Overend, and M. Webb, *Exptl. Cell Research* **2,** 349 (1951).
[155] R. Vercauteren, *Bull. soc. chim. biol.* **32,** 473 (1950).
[156] T. Caspersson, *Skand. Arch. Physiol.* **73,** Suppl. 1 (1936).
[157] T. Caspersson, "Cell Growth and Cell Function." Norton, New York, 1950.
[158] H. G. Davies and M. P. B. Walker, *Progr. Biophys.* **3,** 195 (1953).
[159] E. R. Blout, *Advances in Biol. and Med. Phys.* **3,** 285 (1953).
[160] L. Lison, "Histochimie et cytochimie animales." Gauthier-Villars, Paris, 1953.
[161] A. W. Pollister, *in* "Biological Effects of Radiation" (A. E. Hollaender, ed.), Vol. 2. McGraw-Hill, New York, in press.
[162] A. W. Pollister and L. Ornstein, *in* "Analytic Cytology" (R. C. Mellors, ed.) McGraw-Hill, New York, in press.
[163] T. Caspersson, *J. Roy. Microscop. Soc.* **60,** 8 (1940).
[164] T. Caspersson, F. Jacobsson, and G. Lomakka, *Exptl. Cell Research* **2,** 301 (1951).

been extremely simple in design; a few have been elaborate, and because of expense, and engineering skill required for their construction and maintenance, are available to only a few laboratories.

Some workers, particularly Pollister and Ris,[129] have maintained that information of considerable biological importance could be obtained with simple apparatus, readily available to the average laboratory. This view appears to have been fully justified from the large number of papers which have appeared in the last few years, based on measurements from such instruments. It is unfortunately also true that these instruments have been misused by some. This has resulted in the appearance of a certain amount of conflicting data in the literature, and some natural skepticism as to the validity of cell photometry as a quantitative method.[127,175-177]

Space does not permit discussion of the many technical details encountered in the photometry of nucleic acids. Many of the problems associated with these methods have been adequately treated in the review articles mentioned above. A few points, however, deserve particular emphasis. In many cases adequate calibration methods are much more important than elaborate electronic circuits, and the need for careful testing of instruments cannot be overstressed. In measurements by visible light of Feulgen-stained nuclei, absorption curve analysis is readily carried out[178,179] so that amounts of dye bound can be determined in nuclei of markedly irregular shape or chromatin distribution, provided dye intensity is sufficient.[180] A certain amount of common sense is essential for the photometric study of any tissue. Some nuclei are too pale to give dependable values, or light scatter may be too large. Some nuclei may be too darkly stained and must be measured at wavelengths off the peak absorption. Some are too irregular in outline for accurate volume computations, or the sections may be cut so thin that it is difficult to tell whether a nucleus is whole or cut by the

[165] P. A. Cole and F. S. Brackett, *Rev. Sci. Instr.* **11**, 419 (1940).
[166] G. I. Lavin, *Rev. Sci. Instr.* **14**, 375 (1943).
[167] I. Gersh and R. P. Baker, *J. Cellular Comp. Physiol.* **21**, 213 (1943).
[168] B. Thorell, *Acta Med. Scand.* **129**, Suppl. 200 (1947).
[169] F. M. Uber, *Am. J. Botany* **26**, 797 (1939).
[170] A. W. Pollister and M. J. Moses, *J. Gen. Physiol.* **32**, 567 (1949).
[171] R. C. Mellors, *Science* **112**, 381 (1950).
[172] L. Lison, *Acta Anat.* **10**, 333 (1950).
[173] M. J. Moses, *Exptl. Cell Research* Suppl. 2, 75 (1952).
[174] H. Wyckoff, *Lab. Invest.* **1**, 115 (1952).
[175] B. Commoner, *Science* **110**, 31 (1949).
[176] D. Glick, A. Engström, and B. G. Malmstrom, *Science* **114**, 253 (1951).
[177] H. Naora, *Science*, **114**, 279 (1951).
[178] L. Ornstein, *Lab. Invest.* **1**, 250 (1952).
[179] K. Patau, *Chromosoma* **5**, 363 (1952).
[180] K. Patau and H. Swift, *Chromosoma* **6**, 149 (1953).

microtome. Careful observation of specimens must accompany measurement, and specimens must be prepared with techniques that keep variables at a minimum.

VII. Ultraviolet Absorption

Localization of the purine and pyrimidine bases of nucleic acid through their ultraviolet absorption constitutes one of the most important lines of cytochemical research. The techniques involved, however, are considerably more complex than those required for visible light measurement, and interpretation of the data obtained is more difficult. The quartz or reflecting optical system needed for ultraviolet studies is moderately expensive and often difficult to align and focus. Further, the absorption curves obtained from tissue sections are composites of several components, and the absorption due to nucleic acids alone is often difficult to estimate.

Typical absorption curves on different cell regions are shown in Fig. 7. Where nucleic acid concentration is high (Fig. 7*A*,*B*), the 260-mμ nucleotide peak is prominent. Where nucleotide absorption is low or absent (Fig. 7*D*), the peak at about 280 mμ of tyrosine and tryptophan in the tissue proteins is apparent. Nucleic acid absorption is much stronger than that of proteins; the nucleic acid extinction at 260 mμ is about 40 times higher than the extinction at 280 mμ of a similar concentration of serum albumin. Thus in many cases protein absorption appears merely as a small side deflection on the nucleic acid peak. From the general shape of the absorption curve, however, a qualitative idea of the nucleic acid protein ratio can be obtained.

1. Factors Influencing Absorption Curves

Both nucleic acid and protein curves may change shape under certain conditions. Nucleotide absorption changes only slightly with pH[181] although the long wavelength side of the PNA curve shows an increase in absorption with increasing acidity.[182] Depolymerization of PNA causes a decrease of about 20% at 295 mμ,[182] while depolymerization of DNA results in an increase at 260 mμ of about 30%.[183] Combination of DNA or PNA with some metals ions, such as zinc, causes a shift in the absorption peak to higher wavelengths. Treatment with perchloric acid increases PNA and DNA absorption, and shifts the DNA peak to 268 mμ.[131] Also, the medium in which nucleic acid is dispersed may affect its absorption; the peak for pure aqueous solutions of nucleic acid shifts to longer wavelengths when glycerin is added. A reduction in nucleic acid absorption may occur with prolonged

[181] E. R. Holiday, *Biochem. J.* **24,** 619 (1930).
[182] M. Kunitz, *J. Biol. Chem.* **164,** 563 (1946).
[183] M. Kunitz, *J. Gen. Physiol.* **33,** 349 (1950).

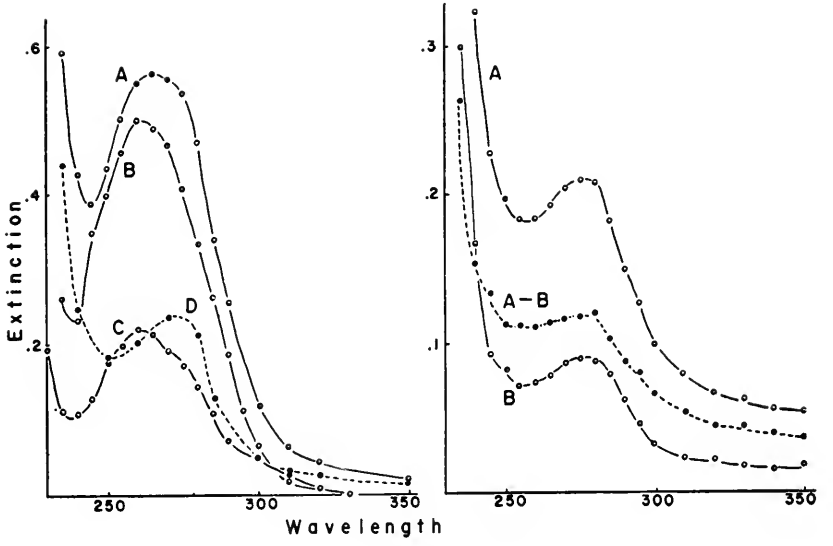


FIG. 7. *Left:* Ultraviolet absorption curves. *A*. Telophase spindle from lily ovary wall, acetic alcohol-fixed, 5-μ section, mounted in zinc chloride-glycerin. *B*. Cytoplasm, *Drosophila* salivary gland cell, smeared and mounted in 50% acetic acid with 1.25% lanthanum acetate as suggested by Caspersson.[187] *C*. Nucleolus of same cell. *D*. Nuclear sap from late salamander (*Triturus*) oocyte, acetic alcohol-fixed, cut at 5 μ and mounted in zinc chloride-glycerine. *Right:* The effects of mounting media. Egg albumen, heat-denatured and fixed in formalin, cut at 5 μ. *A*. Mounted in glycerin. *B*. Mounted in chloral hydrate-glycerin. Curve $A - B$ demonstrates the amount of light lost through scattering; an increased scatter in the region of maximum absorption is evident. The section was first mounted in chloral hydrate-glycerin for curve *B*. The mounting medium was then removed with absolute alcohol (1 hour), the section remounted in glycerin, and the same area was remeasured. Some swelling of the albumin may have occurred in the chloral hydrate-glycerin.

ultraviolet irradiation.[184] In some cases this may involve actual destruction of pyrimidine rings.[185] The effect may be reduced in frozen-dried tissue sections if lanthanum is added to the mounting medium.[184]

Since protein absorption broadly overlaps that of the nucleic acid component, changes in the shape of protein curves are also important for nucleic acid estimation. The tyrosine peak at higher pH levels (above 10) shifts to about 290 mμ and increases in height.[186] A similar shift, originally attributed to the influence of basic proteins, occurs in some tissues;[187] since basic proteins do not show the shift,[188] however, other factors as yet un-

[184] H. K. Catchpole and I. Gersh, *Discussions Faraday Soc.* **9**, 471 (1950).
[185] D. Rapport and A. Canzanelli, *Science* **112**, 469 (1950).
[186] W. Stenstrom and J. Reinhard, *J. Phys. Chem.* **29**, 1477 (1925).
[187] T. Caspersson, *Chromosoma* **1**, 562 (1940).
[188] A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.* **30**, 117 (1946).

known must be involved. A shift to 290 mμ is also caused by certain chemical combinations of the phenolic groups, for example, with mercuric ions. Oxidation of the phenolic group causes marked changes in the shape of protein absorption curves, although the position of the peak is unchanged.[189] Some of these effects on nucleic acid or protein absorption probably do not influence cytochemical determinations; others, however, such as the effect of depolymerization or tyrosine oxidation, may be produced by fixation or embedding of material, and their influence needs investigation, particularly if accurate nucleic acid determinations are desired.

2. Interfering Substances

In addition to protein absorption, there are a number of other cell constituents that absorb in the 260-mμ region, which may interfere with nucleic acid determination. Nucleotides such as ATP or DPN absorp ultraviolet light, but it is doubtful if these occur in sufficient quantity to have noticeable effects on absorption curves of most cells. During the process of fixation and embedding, such compounds are probably readily extracted. In plant cells both catechol and ascorbic acid may add to absorption in the nucleic acid region. Catechol is readily removed by fixation, but ascorbic acid was found to remain in bean root cells after acetic alcohol or absolute alcohol fixation.[190]

A yellow pigment, possibly a pterin, with strongly absorbing 260- and 280-mμ maxima occurs in human nervous tissue. The pigment is not readily extracted and so remains in tissues after cytological preparation.[191] Such substances are probably of little consequence in the great majority of tissues, but should obviously be watched for in any new material. Their presence is usually recognizable where whole absorption curves are run through the region of specific nucleic acid absorption (230 to 320 mμ), and where nuclease-treated blanks are also measured.

3. Nonspecific Light Loss

Nonspecific light loss is particularly severe in the ultraviolet region. Light scattering is produced by the many changes of refractive index in the fine structure of the tissue, and its magnitude increases with smaller wavelengths at a rate dependent upon the dimensions and refractive index of the scattering particles. Since the submicroscopic structure that constitutes living or fixed protoplasm is obviously heterogenous and the particle dimensions are not readily measured, the slope of the scatter curve cannot be experimentally determined. The extent of scatter at 320 to 350 mμ can be

[189] E. S. G. Barron and P. Finkelstein, *Arch. Biochem. and Biophys.* **41**, 212 (1952).
[190] J. Chayen, *Symposia. Soc. Exptl. Biol.* **6**, 290 (1952).
H. Hyden and B. Lindström, *Discussions Faraday Soc.* **9**, 436 (1950).

estimated, however, since nucleic acids and protein have no appreciable absorption in this region (unless tyrosine phenolic groups are oxidized) and most light loss may be considered nonspecific. In some cases Caspersson and his associates have determined the degree of absorption in this area, and then extrapolated the curve into the region of specific absorption.[156,192] As pointed out by many authors,[127,129,158,159] this can only give an extremely rough estimate of the degree of nonspecific light loss. The shape of the scatter curve has been determined by analogy to theoretical systems which obviously are only indirectly applicable.[193,194] Also, where light scatter is produced by absorbing particles, the extent of light loss may be much influenced by anomalous dispersion (see below) so that the amount of scatter is larger in the general region where the specific absorption is greater. For these reasons most of the determinations of nucleic acids that have been made on tissue sections, where marked scatter almost invariably occurs, are best considered qualitative or at most semiquantitative. Techniques for direct measurement of the scattered light have recently been described.[157] This should provide an independent estimate of nonspecific light loss and may greatly simplify its determination.

If ultraviolet absorption measurements on tissues are to be made quantitative, it is obviously of great importance to reduce scatter to a minimum. In most cases glycerin has been used as a mounting medium. This is of much lower refractive index than tissues, and considerable scatter may result from its use, although with frozen-dried material, the nucleoprotein may swell slightly on contact with glycerin and reduce the scatter somewhat.[163] In thin smears, made in acetic alcohol, scattering is also reduced[187] (Fig. 7*B*,*C*), probably in part because a considerable amount of protein is removed. Smears of isolated nuclei show only small amounts of scatter,[195,196] probably also associated with protein loss. Scattering may be practically eliminated where the refractive index of the mounting medium is matched to that of the tissue nucleoproteins. As yet no high refractive index mounting medium has been described that is adequate for ultraviolet studies. Saturated or nearly saturated solutions of chloral hydrate[197] or zinc chloride[198] in glycerin have been suggested and are of the proper refractive index. Both of these media remove nucleic acids from sections after acetic

[192] H. Hyden, *Acta Physiol. Scand.* **6**, Suppl. 17, 1 (1943).
[193] T. Caspersson, *Kolloid-Z.* **60**, 151 (1932).
[194] T. Caspersson, *Kolloid-Z.* **65**, 162 (1933).
[195] C. Leuchtenberger, R. Leuchtenberger, C. Vendrely, and R. Vendrely, *Exptl. Cell Research* **3**, 240 (1952).
[196] R. C. Mellors and J. Hlinka, *Federation Proc.* **12**, 246 (1953).
[197] A. Köhler, *Z. wiss. Mikroskop.* **21**, 273 (1904).
[198] H. Koenig, D. Schildkraut, and E. Galler, *J. Histochem. and Cytochem.* **1**, 384 (1953).

alcohol or Navashin's fixation and consequently they are not suitable for most sectioned material. Zinc chloride further causes a shift of the nucleic acid peak to 270 mμ. Chloral hydrate also removes proteins after acetic alcohol but not after formalin fixation. Absorption curves essentially free from scatter can be obtained by the use of butyl methacrylate as an embedding medium (polymerized by ultraviolet light without plasticizer). Sections up to 5 μ in thickness may be cut with a glass knife, as in preparation of material for electron microscopy. The sections may be spread on slides with 95% alcohol, and mounted in glycerin with the methacrylate still present. Artifacts may be produced, however, by the methacrylate during the process of polymerization. Also it may be hard to remount the section if the methacrylate is once removed, so that sections cannot be measured both before and after they are subjected to enzyme treatment.

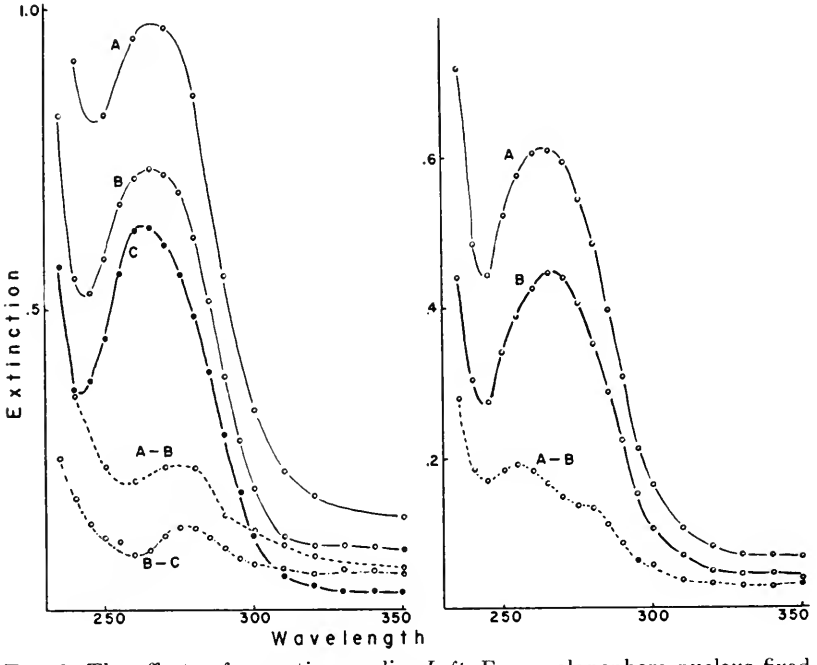


FIG. 8. The effects of mounting media. *Left:* Frog melanophore nucleus fixed in 10% formalin, mounted in butyl methacrylate and sectioned at 5 μ. Section was first measured with embedding medium in place (curve *C*); methacrylate was removed in acetone-xylol, and the section was remounted in glycerin and the same area measured (curve *B*). Curve *A* shows the same area measured in glycerin-water (1:1). The subtraction curves (*A* − *B* and *B* − *C*) show anomalous dispersion primarily of the protein component. *Right:* Frog liver nucleus, prepared as above. *A*. In glycerin. *B*. In methacrylate. Subtraction curve (*A* − *B*) shows both nucleic acid and protein have contributed to nonspecific light loss. The effects are complicated by the slight swelling of tissues in methacrylate.

Although methacrylate refractive index is adequate for some tissues, it is not for others, and is not readily adjusted.

The effect on absorption curves of the refractive index of the mounting medium is shown in Figs. 7 and 8. When the same cell is measured in different media, one of refractive index close to that of the specimen and the other not, an estimate of the light loss due to scatter alone can be obtained. The effect of anomalous dispersion is evident, since the proportion of light lost by scatter is obviously greater in the region of maximum absorption, although the swelling of the tissue in methacrylate may have added further complications. In some cases the effect of anomalous dispersion is greater for the protein component than for the nucleic acid (Fig. 8). Thus scatter effects may easily lead to false interpretations of the nucleic acid–protein ratio, depending upon the degree to which nucleic acid and protein individually contribute to light loss.

From such data it is evident that, if ultraviolet determinations are ever going to provide quantitative estimates of nucleic acids in tissues, high refractive index media should be employed. Where specimens are adequately matched, however, cell details are not readily seen in visible light, so that accurate centration of cell regions is difficult. Fluorescent screens may be used for visualizing the sections, but these provide weak images at best, and the intensities of ultraviolet light required may be harmful to the specimens. Photographic scanning may be used but is tedious. An ultraviolet-sensitive television camera[199] or image intensifier would solve the problem, but these are not as yet generally available.

VIII. Conclusions

Present cytochemical techniques by and large seem to be adequate for the study of many problems concerning the role of nucleic acids in the cell. None of the techniques discussed here, however, can be considered entirely satisfactory, and a number of pitfalls confront the worker in cytochemical research. These difficulties are avoided only by experience with the techniques themselves, a certain skepticism of data rapidly obtained, and a constant checking of specificity and stoichiometry wherever possible. Although a list of steps to follow can be given for many cytochemical methods, for any new material modifications must almost always be made. For example, nuclease enzymes may act rapidly, slowly, or not at all, depending on the type of material and how it is fixed, and may even fail to act specifically under some conditions. Thus a certain amount of empirical manipulation must preface the use of almost any cytochemical method.

Improved cytochemical techniques are needed to extend the types of

[199] G. K. Williams, *Proc. Am. Assoc. Cancer Research* **1**, 60 (1953).

problem that may be approached. There is as yet no adequate method known to the author for chromosomal PNA. A quantitative color reaction would increase our knowledge, as yet fragmentary, of the variations of PNA in interphase nuclei and mitotic chromosomes (see Chapter 20). Although DNA has been considered heterogenous by several workers on the basis of enzyme or solubility studies, no cytochemical tecnhique has been described that would reflect this complexity. Fractionation techniques to distinguish different types of DNA on a cytochemical level would obviously be of great interest. Cytochemistry is a new field, however, and in the space of comparatively few years has already provided the techniques of analysis for hundreds of studies on the biology of nucleic acids. A tremendous number of additional problems await investigation with the methods already at hand.

IX. Methods

The following are intended merely as suggestions for those unfamilar with cytochemical techniques for nucleic acids. Modifications may be necessary with some tissues.

1. Fixation

Neutral formalin: Stock 40% formaldehyde solution 1 part; distilled water, 9 parts with added $CaCO_3$. Small tissue blocks fixed for 1 to 12 hours, followed by washing in water 5 to 12 hours. May be made up in saline for delicate tissues (e.g., chick fibroblasts in tissue culture).

Acetic alcohol: Absolute alcohol, 3 parts; glacial acetic acid, 1 part (must be freshly made). Small tissue blocks 1 to 12 hours, followed by 2 or 3 changes of 95% or absolute alcohol, 1 hour each. Prolonged fixation may decrease basophilia.[31]

Carnoy's fluid: Absolute alcohol, 6 parts; chloroform, 3 parts; glacial acetic acid, 1 part. Treat tissues as for acetic alcohol.

Serra's fluid: Absolute alcohol, 6 parts; stock 40% formaldehyde solution, 3 parts; glacial acetic acid, 1 part. Treat tissues as for acetic alcohol.

2. Extraction

Trichloroacetic acid treatment for nucleic acid removal: 5% trichloroacetic acid at 90° for 5 to 15 minutes. Most acetic alcohol-fixed tissues are readily extracted. Extraction is more difficult after formalin fixation, and often ineffective after fixation with chromic acid fixatives. Treatment should be as short as permissible to avoid protein removal. Protamines are quite readily extracted.

Ribonuclease: crystalline preparations made according to Kunitz[139] or obtained commercially may be used in concentrations from 0.1 to 2 mg./ml. Proteolytic activity may be eliminated by dissolving 5 mg. of the enzyme in 1 ml. of 0.2 saturated ammonium sulfate and placing in a boiling water bath for 5 minutes before making up to desired concentration. The pH may be adjusted to 6 or 6.5 with 0.01 *N* NaOH. Sections may be treated for 1 to 2 hours at 37° (or room temperature). The method works well with acetic alcohol-fixed and formalin-fixed tissues, but high enzyme concentrations are necessary for PNA removal from chromic acid-containing fixatives.

Deoxyribonuclease: Crystalline preparations made according to McCarty,[88] or obtained commercially may be used in concentrations from 0.1 to 1 mg./ml., and adjusted to pH 6.5 with 0.01 *N* NaOH. Magnesium sulfate (0.003 *M*) may be added, but is usually not necessary. Treatment at 37° (or room temperature), for 30 to 60 minutes for most acetic alcohol-fixed plant tissues, and 1 to 2 hours for acetic alcohol-fixed animal tissues, or shorter times with agitation. The enzyme fails to work with many other fixatives; it can be made to work with some formalin-fixed tissues if these are subjected to water at 90° before enzyme treatment.

3. Feulgen Reaction

Feulgen reagent: Add 0.5 g. basic fuchsin (*C.I.* 677) (or pararosaniline, *C.I.* 676) to 100 ml. water at room temperature, add 1 g. potassium (or sodium) metabisulfite and 10 ml. of *N* hydrochloric acid. Shake at intervals until straw-colored (about 3 hours), add 0.25 to 0.5 g. activated charcoal, shake, filter, and store in tightly stoppered bottle in refrigerator. Reagent should be water-clear. If still yellowish add more charcoal and refilter.

Rinse: 10 ml. *N* hydrochloric acid, 10 ml. 5% potassium (or sodium) metabisulfite, 180 ml. water.

Procedure: Hydrolyze sections in *N* hydrochloric acid at 60°. Most acetic alcohol-fixed tissues have a 12-minute optimum, and formalin-fixed tissues 14 minutes. Treat with Feulgen reagent 1 hour, followed by 3 rinses, 10 minutes each (longer for thick sections), wash in water for 5 minutes, dehydrate, and mount. If hydrolysis removes sections from slides, they may be held in place with thin films of celloidon. Run slides to absolute alcohol, dip briefly in 0.5% celloidon solution in absolute alcohol-ether (1:1), allow to dry partially in air for about 15 to 30 seconds, then run slides to water for hydrolysis.

4. Basic Dyes

Azure B:[48] Treat acetic alcohol-fixed tissues for 2 hours at 37° with 0.25 mg./ml. buffered at pH 4.0 (e.g. McIlvaine buffer: 24.6 ml. of 0.1 *M* citric acid to 15.4 ml. of 0.2 *M* disodium phosphate). Rinse slides in water and leave in *tert*-butyl alcohol for 12 hours; mount in balsam or plastic media. Stains DNA blue-green, PNA purple, and other basophilic substances (mucin, chondroitin) reddish.

Methyl green-pyronin: There have been numerous procedures recommended. That of Kurnick[200] gives very satisfactory results with most acetic alcohol-fixed tissues (see also Brachet[31]). Methyl green loses its specificity for DNA in some tissues after formalin fixation. Methyl green (*C.I.* 684) or ethyl green (*C.I.* 685) (0.2%) may be made up in water or acetate buffer (0.1 *M*) at pH 4.1 or 4.2. Solutions should be shaken repeatedly with chloroform in a separatory funnel until the chloroform ceases to show traces of color from crystal violet contamination. Kurnick recommends staining for 6 minutes, although longer times are desirable for some tissues. Blot, and differentiate in two changes of *n*-butyl alcohol (overnight differentiation in *tert*-butyl alcohol has also been recommended[12]). Counterstain in pyronin B (*C.I.* 741) in acetone 30 to 90 seconds, clear in xylene, and mount in balsam or plastic media. Kurnick obtained the best differentiation with *n*-butyl alcohol, which removes pyronin, hence the necessity of separate staining solutions. Many workers have found pyronin unsatisfactory for PNA. Kurnick reports that it "serves primarily as a counterstain and is not found to be a reliable indicator of ribonucleic acid." For this reason Korson[201]

[200] N. B. Kurnick, *Stain Technol.* **27,** 233 (1952).

[201] R. Korson, *Stain Technol.* **26,** 265 (1951).

has replaced pyronin with an aqueous solution of the more dependable toluidine blue O (*C.I.* 925).

Gallocyanine-chrome alum: Stock solution:[61] chrome alum (purified), 5 g. in 100 ml. water, with 0.15 g. gallocyanine, boiled gently for 5 minutes, cooled, and filtered. The pH should be 1.64. The solution slowly deteriorates, and should not be used for more than 4 weeks. A more intense stain may be obtained by increasing the pH, but this also increases the extent of nonspecific staining. Treat slides for 48 hours at room temperature. PNA and DNA are stained a blue-purple (absorption maximum about 500 mμ). There may be some nonspecific background staining.[61-62]

CHAPTER 18

The Isolation and Composition of Cell Nuclei and Nucleoli

ALEXANDER L. DOUNCE*

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* Support of the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, is gratefully acknowledged.

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I. Methods of Isolating Cell Nuclei

1. Methods Involving the Use of Aqueous Solutions

a. Limitations of Methods

The isolation of cell nuclei on a chemical scale can be carried out by methods involving the use of aqueous or nonaqueous media. The use of aqueous media will be considered first, but before the various technical procedures in general use at the present time are discussed, something will be said concerning the limitations imposed by the use of aqueous media.

The first and perhaps the most important limitation is that undoubtedly much low-molecular-weight material is extracted from nuclei during the course of isolation, as well as presumably material of high molecular weight such as protein. The degree of permeability of the nuclear membrane is at the present time a matter of controversy, but in the face of a certain amount of evidence in favor of permeability to material of as high molecular weight as may be exhibited by protein, it is necessary to assume that considerable loss of the latter may occur during the isolation of cell nuclei in aqueous media, in addition to the loss of the unbound low-molecular-weight nuclear constituents. The evidence against a high degree of permeability of the nuclear membrane is at present meager. This subject will be considered in greater detail subsequently, when the composition of nuclei with respect to enzymes is discussed.

The second limitation of major importance is the possibility of adsorption of cytoplasmic material during the isolation procedure, particularly absorption of high-molecular-weight substances such as proteins. The adsorption of soluble protein is not thus far known to constitute a serious difficulty;[1] but the adsorption of very finely divided particulate matter may be serious in certain cases, as will be shown later on. Even if adsorption of a given soluble cytoplasmic protein does not occur on the nuclear surface, there remains the possibility that this protein may form a complex with deoxyribonucleic acid (DNA) and thus be bound by the nuclei. Such a combintion will be caused to dissociate as a rule by the addition of sodium chloride or by adjusting the pH to a sufficiently high value, since nucleic acid and proteins generally form strong complexes only when the pH is above the isoelectric point of nucleic acid (which is very low) and below or close to the isoelectric point of the protein in question.[2,3]

In working with soluble proteins and enzymes, the investigator is therefore always in a dilemma since, if a high pH is used and if sodium chloride is added, it is possible that nearly all of the soluble protein and enzymes originally present within the nucleus may be lost. On the other hand, if the pH is lowered so as to cause binding of intranuclear protein by allowing it to form complexes with DNA, an exchange of nuclear and cytoplasmic protein may still occur to a sufficient extent to be troublesome.[4] In addition to this, the problem of adsorption of insoluble cytoplasmic material must be considered, but such adsorption can be minimized if the nuclei are isolated without concomitant rupture of cytoplasmic granules (mitochondria) so that the latter can be removed from the nuclei while they are still intact. This point will be discussed in detail subsequently.

[1] A. L. Dounce and G. T. Beyer, *J. Biol. Chem.* **174,** 859 (1948).
[2] E. Goldwasser and F. W. Putnam, *J. Phys. and Colloid. Chem.* **54,** 79 (1950).
[3] L. G. Longsworth and D. A. MacInnes, *J. Gen. Physiol.* **25,** 507 (1942).
[4] A. L. Dounce, *Exptl. Cell Research* Suppl. 2, 103 (1952).

One further point which should be mentioned concerning nuclei prepared in aqueous media is that, in the absence of nuclear autolysis, the nuclei should be capable of forming a peculiar type of structural gel upon the addition of alkali or strong sodium chloride. The writer considers gel formation as an indication that little or no autolytic degradation of nuclear structure has occurred.[5,6] If nuclei are isolated from mammalian tissues at pH values ranging from 6 to 7, they often will not form gels. Gels are formed however by nuclei isolated in 70% ethylene glycol or glycerol; in this case the high concentration of organic solvent appears to block autolytic action.

Cell nuclei isolated at a pH of about 6.8 in isotonic saline also can form gels. Here it may be that the use of isotonic saline renders the nucleoprotein complex so insoluble that it is not subject to rapid degradation by autolytic enzymes. It has very recently been found in the writer's laboratory that the use of low concentrations of calcium chloride also can prevent autolytic degradation; more will be said about this later. The loss of ability to form gels, resulting from intranuclear autolysis, may not be of any great significance from the standpoint of the enzyme chemistry of the cell nuclei, but it is interesting in that it demonstrates a type of chemical damage that can occur without corresponding microscopically observable morphological damage.

In spite of the limitations just mentioned, the use of aqueous media for isolating cell nuclei cannot be abandoned. The most important reason for making this statement is that methods involving nonaqueous media can cause damage to or destruction of a number of enzyme systems and therefore are not universally applicable. Such damage or destruction is particularly important in the cases of some of the insoluble mitochondrial enzymes such as cytochrome oxidase and succinic dehydrogenase. In addition, methods making use of aqueous solutions are much less time-consuming than methods involving nonaqueous solvents and can be perfectly reliable for investigations of materials such as lipid and DNA.

b. Methods for Removing Fiber and for Homogenizing Tissue

The first steps in isolating nuclei from tissues on a chemical scale consist in breaking the cells and, where necessary, in removing fiber by special procedures. When nuclei are to be isolated from a relatively nonfibrous organ such as the liver of a young animal, special methods for removing fiber may be dispensed with. It is sufficient in the case of liver to filter the homogenized material through progressively finer grades of cheesecloth,

[5] A. L. Dounce, *Science* **110**, 442 (1949).

[6] A. L. Dounce, G. H. Tishkoff, S. R. Barnett, and R. M. Freer, *J. Gen. Physiol.* **33**, 629 (1950).

such as grades 60, 90, and 120. With small amounts of material, the 60-mesh cheesecloth can be dispensed with. Since 120-grade cheesecloth is only occasionally available, it is usually necessary to substitute some other kind of cloth of the same degree of fineness. Bolting silk is advantageous for the final filtration but is very expensive. Cotton flannel has also been used in our laboratory and elsewhere with considerable advantage, especially in stubborn cases of contamination with fiber and whole cells.

When simple filtrations are used to remove the fiber, it is best not to complete the homogenization until most of the fiber has been filtered off. A preliminary homogenization is effected which is just sufficient to produce a resonably smooth homogenate, free from lumps. However, if bolting silk or cotton flannel is used, the homogenization must be completed before filtration through either of these materials.

Should the organ under examination contain a great deal of fiber, it is necessary to remove the bulk of the fiber before homogenization is begun. To do this, the whole organ, or as large a piece of it as can be used at one time, is placed in a stainless steel cylinder fitted with a piston of the same material and a perforated stainless steel disk through which the tissue can be forced by the application of sufficient pressure. The piston and cylinder fit on the top of a heavy walled stainless steel container designed to catch the tissue as it is forced through the perforated plate. The whole apparatus (see Fig. 1) is placed in a hydraulic press such as the Carver press, and the tissue is forced through the perforated plate at the lower end of the cylinder, leaving most of the fiber as a mat on the upper side of the plate. It is possible to place a stainless steel wire screen on top of the perforated plate to get rid of even more fiber, but in this case the pressure required is very high and some damage may be done to the nuclei. It has been claimed by Hogeboom and Schneider[7] that the use of a device such as has just been described causes some damage to nuclei of liver cells, but nevertheless in work with organs containing a great deal of fiber, it is at least very worthwhile and possibly essential to use the device, since fiber is not readily removed once homogenization has been completed and since too much fiber may even prevent satisfactory homogenization.

The next point to be considered is the technique of homogenization. Much of the early work on the isolation of cell nuclei from mammalian tissues involved the use of the Waring Blendor, with relatively large-scale samples of tissue. The Waring Blendor, or a similar homogenizer, is very useful in preparing relatively large quantities of nuclei for certain purposes, but it has certain serious drawbacks. In the first place, something must be done to reduce the fragility of the nuclei. For this purpose the pH can be

[7] G. H. Hogeboom and W. C. Schneider, *J. Biol. Chem.* **197**, 611 (1952).

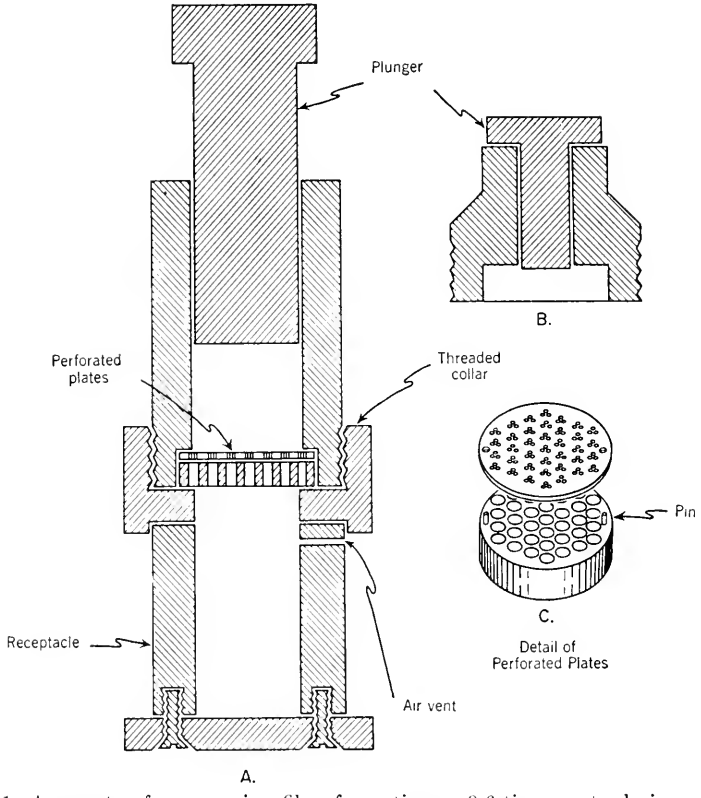

FIG. 1. Apparatus for removing fiber from tissue, 0.6 times actual size; construction of stainless steel. For small quantities of tissue, a smaller top piece and plunger B can be constructed to fit the same perforated plates and collar used for the top piece shown in A. In this case not all the holes in the perforated plates will be used. The larger perforated plate has 37 holes of $\frac{1}{8}$ inch diameter; the smaller upper plate has 3 holes of $\frac{1}{32}$ inch diameter over one $\frac{1}{8}$-inch hole in the larger plate, as shown in A.

lowered to 6 or to 4 or even lower with citric acid;[8-13] or calcium chloride can be added.[14] Otherwise, when the Blendor is run at a high enough speed to break up the cells, the nuclei will also be disintegrated. In the second place, if the Blendor is run at a high enough speed to break up 95% or more of the cells, the mitochondria will also be broken, and, if the Blendor is run at a lower speed, the residual whole cells concentrate with the nuclei

[8] A. L. Dounce, *J. Biol. Chem.* **151**, 221 (1943).
[9] A. L. Dounce, *J. Biol. Chem.* **151**, 235 (1943).
[10] A. Marshak, *J. Gen. Physiol.* **25**, 275 (1941).
[11] F. L. Haven and S. R. Levy, *Cancer Research* **2**, 797 (1942).
[12] A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.* **30**, 117 (1946).
[13] C. Vendrely, *Bull. biol. France et Belg.* **86**, 1 (1952).
[14] R. M. Schneider and M. L. Peterman, *Cancer Research* **10**, 751 (1950).

and render the preparation worthless. Finally, when nuclei are isolated at pH values higher than 4, the Blendor does destroy a considerable proportion of nuclei even under the best conditions obtainable, so that the yields are low. In spite of these drawbacks, nuclei can be isolated from liver tissue at pH 4 or slightly lower in good yield and in a condition remarkably free from microscopically visible impurities. Such nuclei are quite suitable for studies of nuclear lipid, DNA, nuclear PNA, and histone, or for analytical studies of the nonhistone nuclear protein, much of which may however be denatured. These nuclei are not in general suitable for enzyme studies mainly because the pH obtaining during the isolation is so low that many or most enzymes are rendered inactive.

One of the worst drawbacks in the use of the Waring Blendor for preparing nuclei at pH 6.0 or above for use in enzyme work is that the mitochondria are broken, and adsorption of microscopically invisible mitochondrial fragments on the nuclear surfaces can cause serious contamination of the nuclei with mitochondrial enzymes. The somewhat low yields of nuclei obtained when the Waring Blendor is used are not particularly troublesome since, if a sufficient amount (50 g.) of tissue is used, enough nuclei are obtained for many enzyme analyses. A final drawback to the use of the Waring Blendor is that it must be run for a considerable length of time to achieve proper breaking of the cells, and temperature control therefore becomes a problem.

Another device that can be used in the preparation of cell nuclei on a large scale is the colloid mill,[14a] such as that sold by the Premier Mill Company of Geneva, New York. (Models of several sizes are available.) The colloid mill consists essentially of a short truncated stainless steel cone which can be rotated at very high speed inside a second fixed stainless steel cone. These cones are machined to fit each other very exactly. The material to be homogenized is fed into the mill by gravity through a stainless steel funnel and, since the cones are placed with axes vertical and bases downward, the centrifugal force which is developed in the liquid layer between the cones causes the liquid to be fed downward at a reasonably high speed, which depends upon the spacing between the cones. The latter may be adjusted over a wide range, but clearance of 1- to 2-thousandths of an inch or under certain circumstances even less are generally used. A medium or high speed (attainable by changing pulleys) is in general preferable to a low speed, but in the writer's laboratory a medium speed (10,000 r.p.m.) has been found to give best over all results, with a cone clearance of 1.5-thousandths of an inch. The funnel of the mill can be chilled to 0° before use by being filled with crushed ice or by being placed in the cold room.

[14a] The colloid mill was introduced in cytochemistry by Mirsky for the isolation of chromosomes.

The colloid mill acts mainly by creating high shearing forces in the liquid medium, and is more or less free from the effects of mechanical battering such as is caused by the blades of the Waring Blendor. No doubt some mechanical effects occur however, since the flow is undoubtedly turbulent owing to the high speed of rotation, and some if not all of the cells must strike the surfaces of the cones with considerable force.

One great advantage of the colloid mill is that a large quantity of homogenate may be very rapidly processed, since a few minutes usually suffice to run the material through the mill once, and two or at the most three passages through the mill suffice to give practically complete cell breakdown under favorable conditions.

Another advantage of the colloid mill is that it has a less pronounced tendency to disintegrate the nuclei than has the Waring Blendor. Moreover it will rupture whole cells more efficiently than does the Waring Blendor, so that it is possible to obtain preparations of nuclei in cases where the cells are so resistant that the Waring Blendor will not break them or will do so only with simultaneous breaking of the nuclei.

The colloid mill is however, not without several disadvantages. In the first place, some sort of preliminary homogenization has to be carried out in order to get the material into a sufficiently liquid state to be run through the mill. As a rule a very short treatment in the Waring Blendor is satisfactory for this purpose (about 1 minute, with a rather low speed). When this procedure is followed, it is best to strain the material to remove fiber before using the colloid mill, but the finest cloth (cotton flannel or bolting silk) should not be used until the material has been passed through the colloid mill.

Another disadvantage of the colloid mill is that, like the Waring Blendor, it disrupts mitochondria. As already has been noted, this is not desirable if the nuclei are to be used for enzyme studies.

Still another disadvantage is that the resistance of the nuclei to disintegration must be increased by adding calcium chloride or lowering the pH as is done when the Waring Blendor is used. Such procedures also as a rule increase to some extent the resistance of the whole cells to breakdown, as has already been mentioned.

In spite of the disadvantages just cited, the colloid mill is very useful in preparing large quantities of cell nuclei from organs such as liver or pancreas, and this is of considerable importance when it is desired, for instance, to proceed further to isolate nucleoli from the isolated nuclei, or to prepare relatively large amounts of lipid, protein, or nucleic acid from nuclei. A cheaper type of hand-operated colloid mill has been used by Denues[15] in the isolation of chromosomes.

[15] A. R. T. Denues, *Exptl. Cell Research* **3**, 388 (1952).

A third recently described homogenizer for making homogenates on a somewhat smaller scale than with the Waring Blendor or colloid mill is the stainless steel apparatus described by Lang and collaborators.[16]

Apart from various other high-speed mixers, which undoubtedly disrupt mitochondria,[14,17] the devices just described constitute the principal means known at the present time for preparing homogenates to be used in isolating cell nuclei on a relatively large scale. It is possible that a device such as the Latapie homogenizer might be useful in certain instances, but the writer has had no experience with this apparatus. In the disruption of starfish egg cells prior to the isolation of nucleoli, homogenization has been accomplished by forcing a cell suspension through a hypodermic needle.[18]

The best-known small-scale homogenizer which can be used for obtaining homogenates useful in isolating cell nuclei on a small scale is the ground-glass apparatus now known as the Potter-Elvehjem homogenizer.[19] A device built on exactly the same principle was first described by Hagan in 1922,[20] as noted by Potter.[21] The Potter-Elvehjem homogenizer was constructed in a different manner and the slight modification of fusing small glass beads on the bottom of the pestle was introduced, but the final product was of practically the same design as the one described by Hagan.

The Hagan-Potter-Elvehjem homogenizer has been used extensively by Schneider and Hogeboom and their followers in preparing homogenates from which cell nuclei are to be obtained. It has two inherent advantages, one of which is the forcing of all of the homogenate between the homogenizing surfaces every time the ground test-tube is run up or down the rotating pestle. The other is a relative gentleness of action which leaves the mitochondria in an undamaged or only very slightly damaged condition, provided that the proper homogenizing medium is used. The nuclear membranes also appear to escape damage to a considerable extent. Certain disadvantages are found, however, when this instrument is used for homogenizing tissue preparatory to the isolation of nuclei, of which the most serious is the failure to break a sufficiently high percentage of the cells. Residual whole cells tend to concentrate with nuclei and can be removed only by special methods such as gravity sedimentation in a two-section cell[1] or filtration through cotton flannel.[1,14,22] But if nuclei are heavily contaminated with whole cells, it is practically impossible to separate them.

[16] K. Lang and G. Siebert, *Biochem. Z.* **322,** 360 (1952).
[17] K. S. McCarty, *J. Exptl. Med. and Surg.* **7,** 213 (1949).
[18] W. S. Vincent, *Proc. Natl. Acad. Sci. U.S.* **38,** 139 (1952).
[19] V. R. Potter and C. A. Elvehjem, *J. Biol. Chem.* **114,** 495 (1936).
[20] W. A. Hagan, *J. Exptl. Med.* **36,** 711 (1922).
[21] V. R. Potter, *J. Biol. Chem.* **163,** 437 (1946).
[22] G. H. Hogeboom, W. C. Schneider, and M. J. Striebich, *J. Biol. Chem.* **196,** 111 (1952).

A second drawback of the Hagan-Potter-Elvehjem instrument is that an unknown degree of local heating by friction may occur at the grinding surfaces when homogenization is protracted, and this friction also may cause a small amount of ground glass to be introduced into the homogenate. In the experience of the writer, the Hagan-Potter-Elvehjem homogenizer, which by universal agreement is excellent for work in isolating mitochondria and microsomes, is not a good homogenizer for work in isolating cell nuclei.

A small-scale homogenizer has recently been devised in the writer's laboratory which can be used for preparing cell nuclei or mitochondria and which, for soft tissues, is superior to any homogenizer described in the literature in sufficient detail to be easily constructed. This homogenizer functions best with relatively soft tissue such as liver or pancreas, but it could possibly be used on tougher tissue if the latter were first squeezed through small openings, as in the apparatus shown in Fig. 1.

The homogenizer is constructed of heavy-walled glass tubing and glass rod. The tubing is ground cylindrical on the inside. Precision-bore glass tubing would presumably be an excellent substitute for the ground tubing if it could be obtained with sufficiently thick walls. A bulb blown near the top of the tube acts as a reservoir. The pestle is constructed of heavy glass rod with a round ball on the bottom end and a short thick crosspiece at the top for grasping with the hand. The ball is made very slightly oversize and is fitted to the cylinder by grinding with fine carborundum (number 400 or number 600). After grinding, the ball is cautiously fire-polished to aid in avoiding excessive wear. The homogenizer is inserted in a hole in a large rubber stopper which serves as a base, and is immersed in a bath of ice and water before use. The operation is manual, the pestle being simply pushed down and pulled up without rotation. It is a good plan to start the homogenization with a rather loosely fitting pestle, using about half a dozen strokes, and then, after filtering to remove fiber, to change to the more exactly fitting pestle which should operate with a clearance in the neighborhood of 0.5-thousandths of an inch or slightly less. From six to thirty-six strokes of the well-fitting pestle may be required for complete homogenization, depending upon the tissue and the medium used. Since this requires considerable effort, it might be desirable to fit the plunger with a lever, but we have not found it necessary to do so. The barrel of the homogenizer should be filled not quite to the bottom of the bulb for best results. A diagram of this homogenizer is shown in Fig. 2. A similar homogenizer no doubt could be built on a considerably larger scale from stainless steel.

The principal advantage of the homogenizer just described is that a tight fit may be obtained between the ball and cylinder wall without rendering the plunger immovable. This is because the space between the ball and cyl-

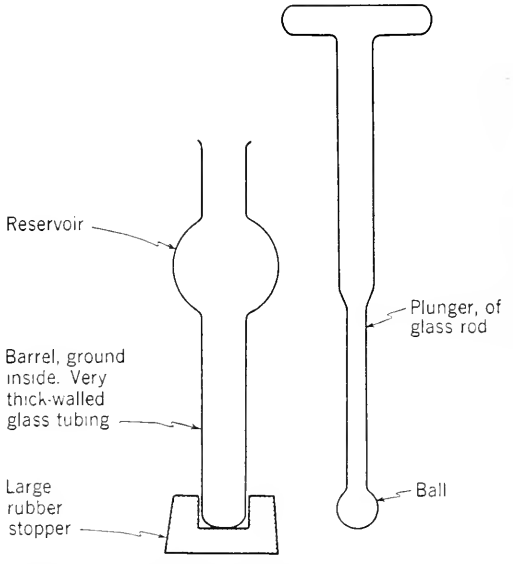

FIG. 2. Ground-glass homogenizer, 0.25 times actual size. Construction probably could also be in stainless steel, particularly if larger sizes were to be made.

inder is occupied entirely by a circle of liquid, so that the forces in the liquid are not too great to be overcome when the plunger is moved. If a homogenizer of the Hagan-Potter-Elvehjem type were constructed with an equally good fit, it would behave like a hypodermic syringe, in that the plunger could not be driven through the liquid in the cylinder.

Other advantages of the new homogenizer are that the friction is reduced to a minimum so that no appreciable heating can occur, and no ground glass accumulates. The relatively tight fit makes it possible to break a very high percentage of the cells present, and yet the nuclei are only slightly damaged. Mitochondria may not be damaged to a detectable extent.

A hand-homogenizer which might resemble the one just described has been mentioned in the literature,[23] but the description of this instrument is so meager that it is impossible to know whether it is similar or not. Wilbur and Skeen[24] have described a hand-operated homogenizer with a pestle made of a glass rod, the lower end of which is covered with a piece of tapered gum rubber tubing.

c. Perfusion of Organs for the Removal of Blood

A topic which deserves some consideration in a description of methods for isolating cell nuclei is the perfusion of organs to remove blood. If the most

[23] H. Stern, V.G. Allfrey, A. E. Mirsky, and H. Saetren, *J. Gen. Physiol.* **35,** 559 (1952).
[24] K. M. Wilbur and M. V. Skeen, *Science* **111,** 304 (1950).

gentle methods available are used for breaking cells, whereby mitochondria or nuclei are not disrupted, the erythrocytes present in the tissue will also remain unbroken and will concentrate in the nuclear fractions. It is therefore necessary either to eliminate the red cells by some step which causes them to become laked or else to remove them by perfusing the organ from which the nuclei are to be isolated. Perfusion with isotonic saline at 0 to 5° until the organ ceases to lose color, followed by perfusion at the same temperature with 0.25 *M* sucrose solution containing calcium chloride at a concentration of 0.0018 *M* as advocated by Hogeboom and Schneider,[7] constitutes a satisfactory procedure for liver and no doubt for other organs also. It is essential to use the isotonic saline before the sucrose, since the latter solution alone will not wash out all the blood. In the case of liver, perfusion through the portal vein is satisfactory. To avoid difficulty in cannulation, the cannula should be inserted in the vein and tied before the liver is removed from the animal.

d. Description of Methods

It would require too much space to go into the details of all methods that have been published for isolating cell nuclei in aqueous media and the reader is referred to the papers cited. Certain methods which have been used in the writer's laboratory and which seem to be of rather general utility will however be covered in some detail.

(1) *Methods Involving the Use of Citric Acid in Water Solution.* The isolation of cell nuclei from rat liver at pH 4.0 or slightly lower[9] is an example of a method which can be used on a relatively large scale to obtain sufficient quantities of nuclei for the subsequent isolation of nuclear constituents such as lipid and DNA, as has already been stated. Homogenization is achieved either by the Waring Blendor alone or by a short treatment in the Waring Blendor followed by passage through the colloid mill. Water is used as suspending medium and the pH is adjusted with molar citric acid. Fifty grams of frozen, chopped-up rat liver is placed in a Waring Blendor with 200 ml. of ice-cold water. Seven milliliters of molar citric acid is added and the Blendor is run for about 1 min. at full or slightly reduced speed. The mixture is then filtered once through Curity cheesecloth grade 60, 4 layers; twice through grade 90, 4 layers; and twice through grade 120, 4 layers. The pH is then checked with the Beckman pH meter using a small sample of homogenate that has been removed for this purpose and allowed to warm up to room temperature. In carrying out the preliminary homogenization, the Waring Blendor is operated in the cold room at 0 to 3° and all filtrations are also carried out in the cold room. It is desirable to add a very small amount of cracked ice to the Blendor and also to add pieces of cracked ice to the portion of homogenate which is filtering through the cheesecloth and to the portion in the collecting beaker. The addition of too much ice added to the Blendor should be avoided since it causes the formation of an unmanageable slush.

The filtered preliminary homogenate can now be treated in one of two ways. It can be put back into the Waring Blendor for 7 to 8 min. with the Blendor operating at a reduced speed obtained by the use of a rheostat to lower the applied voltage to

95 volts a.c.; or it can be run through the colloid mill which is operated at medium speed (10,000 r.p.m.) with a clearance of about 1.5-thousandths of an inch. After the final blending there will be considerable foam which tends to entrap fine fiber as it rises to the top. By pipetting the homogenate from beneath the foam, fiber may be conveniently removed. The temperature must be kept as close to 0° as possible, and the addition of ice from time to time during the blending is desirable if the Waring Blendor is used for the final homogenization. If the colloid mill is used, a mixture of ice and water is placed in the funnel, and ice-water is used to wash the last of the liver homogenate through the mill, since the mill rotor continues to spin for some time after the current has been turned off and the mill must not be run dry. It is convenient to have two persons operating the colloid mill and two passages of the homogenate through the mill are sufficient.

The isolation of the nuclei from the homogenate by differential centrifugation has been adequately described in the original reference.[9]

If nucleic acids or lipids only are to be studied, it may be permissible to isolate the nuclei in strong citric acid which facilitates the rapid preparation of very clean nuclei from a wide variety of tissues and even from tumor tissues where milder methods usually fail. Such methods are described in references 10, 11, 12, and 13 and similar methods have also been published by Barnum *et al.*,[25] by Mirsky and Pollister,[12] and by Frazer and Davidson.[26]

The method of isolation of nuclei at pH 5.9 to 6.0 is similar to that just described except that more dilute citric acid is used. In the original method[8] chopped frozen liver was added to ice-cold very dilute citric acid in the Waring Blendor. In a subsequent modification,[27] the liver was blended for a very short time in ice-water in the Blendor and citric acid was then added dropwise. For reasons outlined elsewhere, we now believe it is preferable to add the liver to the dilute citric acid in the Blendor, and after a short mixing (of about 1 min.) to filter as described above. Homogenization is then completed by operating the Waring Blendor at 95 volts a.c. for 7.5 min. for 50 g. of liver (15 min. for 100 g.); or the colloid mill may be used as described for nuclei prepared at pH 4.0. If the liver is to be added to the citric acid solution as now recommended, 50 g. of frozen liver is added to 200 ml. of ice-cold water containing 5.6 ml. 0.1 *M* citric acid. Since this amount of acid will lower the pH to 5.9 to 6.0, the procedure is safer than the use of a pH range of 6.0 to 6.2 as originally recommended, since fewer nuclei will be disintegrated. A little more acid may be added as required to adjust the pH to 5.9; it should not be allowed to rise above 6.0.

The centrifuging schedule is similar to that used for preparing nuclei at pH 4.0, and the final washings are carried out in the same way. The nuclei may be transferred to a smaller centrifuge tube for the last two or three washings.

(2) *Methods Involving Solutions of Gum Arabic in Water.* The isolation of nuclei at pH 5.9 to 6.0 is greatly facilitated by the addition of ice-cold 5% gum arabic solution (previously adjusted to pH 6.0) to the homogenate just before the first centrifugation.[28] Enough of the gum arabic solution is added to make the final concentration of gum 1%. Subsequent washings are carried out with 1% gum arabic solution adjusted to pH 6.0. The addition of gum arabic also tends to lessen the adsorption of fragmented mitochondria by the nuclei.

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[25] C. P. Barnum, C. W. Nash, E. Jennings, O. Nygaard, and H. Vermund, *Arch. Biochem.* **25,** 376 (1950).

[26] S. C. Frazer and J. N. Davidson, *Exptl. Cell Research* **4,** 316 (1953).

[27] A. L. Dounce, *Ann. N. Y. Acad. Sci.* **50,** 982 (1950).

[28] A. L. Dounce and M. Litt, *Federation Proc.* **11,** 203 (1952).

Usually one original centrifugation and three washings are sufficient to produce clean nuclei. In the last two washings, the pH of the gum may be as high as 6.2. Gum ghatti adjusted to pH 6.0 can also be used instead of gum arabic.[29] It should be noted however that it is not possible to use gum arabic in isolating cell nuclei at pH values of 4.0 or less, since in these conditions the gum forms complexes with the particulate matter in the homogenates and causes massive agglutination.

(3) *Methods Involving the Use of Sucrose in Water Solution.* It is possible to isolate liver cell nuclei on a relatively large scale using isotonic sucrose solution as suspending medium, provided that the pH is lowered to 5.9 to 6.0 before homogenization in the Waring Blendor or colloid mill.[30,31] Such nuclei are very similar in properties to those isolated in water or gum arabic solution with the pH lowered to 5.9 to 6.0. Since no gel is formed on adding alkali or sodium chloride to the nuclei isolated in sucrose at pH 6, it would appear that an autolytic degradation has presumably occurred.

It is also possible to isolate liver cell nuclei in isotonic sucrose solution to which calcium chloride has been added. Calcium chloride was introduced by R. Schneider and Peterman[14] in isolating cell nuclei, and its use has been taken up by Hogeboom *et al.*[22] in the development of a small-scale method for isolating liver cell nuclei. At Rochester, calcium chloride-sucrose solutions have been found very suitable in large- and small-scale isolations of liver cell nuclei. A point of major interest is that calcium chloride apparently inhibits autolytic changes and permits the isolation of liver cell nuclei at pH values sufficiently high to allow the formation of gels with alkali or saline. Concentrations of calcium chloride as low as 0.0018 *M* suffice under certain conditions for this purpose, but higher concentrations are desirable for obtaining clean nuclei. On a "macro" scale the following procedure is recommended.

Fifty grams of frozen rat liver is mixed for about a minute in the Waring Blendor with 250 ml. of 0.25 *M* (isotonic) sucrose solution containing calcium chloride in a concentration of 0.005 *M*. Filtration through cheesecloth as described previously is carried out and the filtered preliminary homogenate is then passed twice through the colloid mill at medium speed (10,000 r.p.m.) with a clearance of 1.5-thousandths of an inch. The homogenate is poured into two 250-ml. centrifuge bottles and underlaid with about 100 ml. of 0.0002 *M* $CaCl_2$-0.34 *M* sucrose solution. The homogenate thus obtained is centrifuged at about 2200 r.p.m. for 20 min. The supernatant is poured off, care being taken not to lose too much loosely packed sediment, and the sediment is then suspended with thorough shaking in about 200 ml. of 0.25 *M* (isotonic) plain sucrose solution (without the addition of any calcium chloride). The pH is then adjusted to 6.2 with dilute citric acid (about 0.01 *M*), care being taken not to go below the desired pH value. (A small sample of the material is warmed to room temperature when testing the pH.) The suspension is then placed in two portions in two 250-ml. centrifuge bottles and each portion is underlaid with about 100 ml. of 0.34 *M* plain sucrose solution. The material is centrifuged for 30 min. at 1500 r.p.m. The supernatant is discarded and the sediment is suspended in about 100 ml. of 0.25 *M* (isotonic) sucrose solution, no pH adjustment being necessary. The material is placed in a 200-ml. centrifuge bottle and is underlaid with 0.34 *M* sucrose solution. Centrifugation is carried out at 1300 r.p.m. for 30 min.

The supernatant is poured off and the sediment is suspended in about 150 ml. of 1% gum arabic solution which has been previously adjusted to pH 6.2. The nuclei are centrifuged down for 20 min. at 1300 r.p.m. The supernatant is discarded and a

[29] E. R. M. Kay, Ph.D. Thesis, University of Rochester, 1953.

[30] K. Arnesen, Y. Goldsmith, and A. D. Dulaney, *Cancer Research* **9**, 669 (1949).

[31] A. L. Dounce, *J. Cellular Comp. Physiol.* **39**, Suppl. 2, 43 (1952).

second washing is carried out with 1% gum arabic solution. If desired the nuclei can be suspended in gum arabic solution, sucrose solution, or distilled water. Two or three quick washings with water or sucrose solution are necessary to remove gum arabic, but it is not possible to remove all traces in this way. Gum arabic subsequently interferes in the colorimetric determination of PNA.

In these procedures all solutions used are ice cold, and all centrifugations are carried out in a refrigerated centrifuge. Care is taken not to allow much warming of the homogenate during filtrations. It is desirable to work in the cold room if possible. The adjustment of the pH to 6.2 with citric acid without the addition of more $CaCl_2$ prevents the clumping of mitochondria and nuclei which occurs in the procedure of Hogeboom and Schneider. Treatment with gum arabic solution removes erythrocytes (by laking).

The method just described may also be carried out on a small scale, using the improved homogenizer already mentioned, with the particular advantage that mitochondria can be removed while still intact, so that contamination of the nuclei by adsorbed mitochondrial fragments is avoided. The nuclei appear spherical or slightly deformed, still possess the nuclear membrane, and are of a hyaline appearance. They are quite clean microscopically and almost free from the cell membranes mentioned by Hogeboom *et al.*[22] They are also obtainable in reasonably good yield, but it is unlikely that any method at present known is capable of producing clean nuclei in very high yield, despite claims to the contrary.

The adaptation of the above procedure to small-scale work furnishes a method for isolating cell nuclei which is believed to be superior even to the latest method of Hogeboom *et al.*[22] The chief drawback to the method of Hogeboom *et al.* is that, if calcium chloride is added during the washing, enough agglutination of the mitochondria and cell membranes is caused to make complete separation of these contaminants very difficult or impossible, at least by the technique of differential centrifugation. Lowering of the pH to 6.2 after the first homogenization in the procedure described in this chapter obviates this difficulty and at the same time prevents the agglutination of the nuclei themselves, but the procedure admittedly incorporates certain steps used by Hogeboom *et al.*[22] and is based on the discovery by R. Schneider and Peterman[14] of the beneficial effects of $CaCl_2$.

The details of the procedure as adapted to small-scale work with rat liver are as follows. Two young or medium-sized rats are killed by decapitation and the livers are dissected out. After removal of as much as possible of the connective tissue and large veins by further dissection, the livers are weighed and then gently pounded to a pulp in an ice-cold mortar. This pulp is mixed with a volume of homogenizing liquid (0.25 *M* sucrose containing 0.005 *M* $CaCl_2$) equal to five times the weight of the livers. The pulped liver is suspended as well as possible and is homogenized in the ball-type homogenizer (Fig. 2) with a rather loosely fitting pestle (0.1- to 0.2-thousandths of an inch clearance). In this step the plunger is raised and lowered six times. The bottom of the homogenizer rests in a large rubber stopper with a hole bored part way through to receive the end of the homogenizer tube. The homogenizer and rubber stopper are immersed in a large beaker containing a mixture of ice and water for cooling.

After this preliminary treatment, the homogenate is filtered once through curity grade 90 cheesecloth and twice through 120-grade cheesecloth or other similar fabric. The filtered homogenate is then homogenized in the ball-type homogenizer using a well-fitting ball (0.5-thousandths of an inch or slightly less in clearance). About 18 to 24 strokes are generally required to break a sufficiently high proportion of the cells, since the calcium chloride renders the cells somewhat resistant to breaking. The progress of the homogenization can be followed by means of the microscope, using the 4-

mm. objective, to a point where the cells become so rare that they have to be hunted for. Excessive homogenization is to be avoided, since it tends to damage nuclei and lower the yield. If a small homogenizer is used, it may be necessary to homogenize the material in two or three batches.

The final homogenate after being divided into two portions is underlaid with approximately equal volumes of 0.34 *M* sucrose solution containing $CaCl_2$ in 0.0002 *M* concentration, in two 60-ml. centrifuge tubes. Centrifugation is carried out for 10 min. at about 2900 r.p.m.

The supernatant is discarded up to, but not including, the loosely packed sediment. The sediment is suspended in about 30 ml. of 0.25 *M* sucrose *without* $CaCl_2$ and after complete suspension by homogenizing with 3 strokes in the loosely fitting homogenizer, the pH is carefully adjusted to 6.2 by the addition of 0.01 *M* citric acid. The suspension is divided into two equal portions, each of which is underlaid with an equal volume of 0.34 *M* sucrose *without* calcium chloride, again using 60-ml. centrifuge tubes. Centrifugation is carried out for 10 min. at 2100 r.p.m.

The supernatant is poured off and the nuclei are again carefully suspended (using the loosely fitting homogenizer with 3 strokes of the plunger) in about 25 ml. of 0.25 *M* sucrose without calcium chloride. No adjustment of pH is necessary. The resulting suspension is underlaid by an approximately equal volume of 0.34 *M* sucrose without calcium chloride, using one 60-ml. centrifuge tube. Centrifugation is carried out for 10 min. at 1800 r.p.m.

The supernatant is discarded, leaving nuclei which are now almost free from mitochondria but which contain a relatively large admixture of erythrocytes. If an appreciable number of mitochondria persist, another one or two centrifugations can be carried out in sucrose solution. Usually this is not necessary, and, since the nuclei must be suspended in 0.25 *M* sucrose to search for mitochondria, this extra centrifugation is omitted except in cases where very exacting work is required.

The sediment of nuclei and erythrocytes is next suspended in about 50 ml. of 1% gum arabic solution previously adjusted to pH 6.2. Centrifugation is carried out for 15 min. at 1800 r.p.m., and the supernatant is checked microscopically to be sure that most of the nuclei have been sedimented. The supernatant is discarded and the nuclei are washed once more in about 15 ml. of 1% gum arabic solution in a 15-ml. centrifuge tube, the centrifuge being run for 15 min. at 1200 r.p.m. The gum should be subsequently removed by washing the nuclei with distilled water if its absence is essential for the work in hand. If the livers are perfused as described previously, the use of gum arabic can be dispensed with entirely.

It is also possible to isolate nuclei from rat liver by a procedure nearly identical with the above but in which 0.88 *M* (30%) sucrose is substituted for isotonic sucrose. In this case the mitochondria maintain their usual shape and possibly suffer less physical damage than when isotonic sucrose is used. If should be kept in mind that the less the damage to mitochondria, the less is the likelihood of loss of mitochondrial enzymes to the supernatant with possible subsequent transfer to nuclei. Since nuclei isolated in this way from 0.88 *M* sucrose are more deeply colored than those isolated in 0.25 *M* sucrose, it is possible that the concentrated sucrose causes adsorption of colored material, e.g., hemoglobin from the supernatant. Perhaps some intermediate concentration of sucrose might prove more suitable than either 0.25 *M* or 0.88 *M*.

(4) *Methods Involving Aqueous Solutions of Ethylene Glycol, Glycerol, or Polyethylene Glycol.* Nuclei can be isolated in solutions containing ethylene glycol or glycerol at *final* concentrations of about 70%. The use of glycerol was first reported by R.

Schneider[32] and the use of ethylene glycol by Mazia.[33] Dallam and Thomas[34] have published a method for isolating cell nuclei with glycerol. The combined use of the Waring Blendor and the colloid mill or the new ball-type homogenizer can be employed for homogenization. If the Waring Blendor and colloid mill are used, mitochondria are disintegrated.

Complete descriptions of methods for isolating cell nuclei with ethylene glycol or glycerol are lacking and the reviewer has not worked out isolation procedures in great detail. The general procedure is to homogenize one part by weight of fresh liver with 5 parts by volume of 80% ethylene glycol or glycerol. The final concentration of solvent in such a homogenate is about 70%. Filtration through cheesecloth is difficult and centrifugation of the nuclei requires considerably longer than with the methods previously described. The once-centrifuged nuclei are then washed three or four times with 70% ethylene glycol or glycerol, care being taken to centrifuge long and hard enough to avoid excessive losses of nuclei.

The nuclei thus obtained are capable of forming gels with alkali and hence have presumably been protected from autolysis by the high concentration of organic solvent. They are rather swollen and of a reddish color which is due to the presence of some adsorbed hemoglobin. Some whole cells are apt to be present, since it is difficult to break all of the cells in ethylene glycol or glycerol without excessively lowering the yield of nuclei. The yield as a rule tends to be low, but with some practice it is possible to obtain reasonably good quantities of nuclei with the large- or small-scale variation of the method. The reviewer prefers ethylene glycol to glycerol, although either solvent can be used.

Polyethylene glycol has been recommended by McClendon and Blinks[35] for use in the isolation of plastids from plant cells. Although this material does not appear to have been tried in the isolation of cell nuclei, it might well furnish a good medium.

Pectin has been used in the isolation of plant cell nuclei by Brown.[36] This is presumably another illustration of the use of a high-molecular-weight colloid for the purpose of increasing the viscosity of the solution so as to allow better separation of the nuclei. Other materials that might possibly be useful are dextran and polyvinylpyrrolidine.

(5) *Methods Involving the Use of Buffered or Unbuffered Salt Solutions.* It is possible under certain conditions to isolate cell nuclei in buffer or saline solutions. In general this cannot be done in a satisfactory manner with the Waring Blendor or colloid mill, but in certain cases it can be achieved with ground-glass hand-homogenizers. A serious drawback to the use of saline or buffers is that the mitochondria tend to agglutinate, and hence, if any purification is to be achieved by differential centrifugation, very dilute homogenates must be used and the homogenizer must be used to resuspend the nuclei after each centrifugation. Dilute buffers are sometimes used in conjunction with sucrose solutions, as advocated by Wilbur and Anderson.[37]

Using 0.9% NaCl solution in homogenization and in all subsequent steps, the reviewer has been able to isolate fairly pure nuclei from normal rat liver. The ball-type

[32] R. M. Schneider, *Federation Proc.* **11**, 140 (1952).

[33] D. Mazia, *in* "Trends in Physiology and Biochemistry" (Barron, ed.), p. 99. Academic Press, New York, 1952.

[34] R. D. Dallam and L. Thomas, *Federation Proc.* **12**, 193 (1953).

[35] J. H. McClendon and L. R. Blinke, *Nature* **170**, 577 (1952).

[36] R. Brown, *Nature* **168**, 941 (1951).

[37] K. M. Wilbur and N. G. Anderson, *Exptl. Cell Research* **2**, 47 (1951).

homogenizer was used for breaking the cells, and care was taken to keep the nuclear suspensions quite dilute, especially in the last stages of purification, in order to overcome agglutination of the mitochondria. These nuclei formed gels with alkali. No work on enzyme content has yet been done. Liver cell nuclei also have been isolated on a larger scale in 0.9% NaCl to which $CaCl_2$ had been added.[31] The latter nuclei contained a higher percentage of DNA than nuclei isolated in very dilute citric acid solution at pH 6.0. This is not surprising, since physiological saline solution extracts protein from these nuclei as well as from the sucrose-calcium chloride nuclei prepared by the more recent procedure. Nuclei prepared by the latest published procedure of Hogeboom and Schneider[22] also lose protein when extracted with 0.9% NaCl solution. In general, buffers or saline solutions tend to extract protein from cell nuclei, and in addition they often tend to increase the resistance of whole cells to breakage.

A number of methods have been outlined above for obtaining cell nuclei in aqueous media. These methods all have been applied to liver cells, but they are not all universally applicable. Kidney and pancreas are reasonably similar to liver in behavior, but thymus differs. The original method for isolating cell nuclei at pH 6 in very dilute citric acid is applicable to liver, kidney, and pancreas provided that steps are taken to remove fiber where necessary, but generally it will not work with thymus. It is quite likely that the calcium chloride-sucrose method discussed above would be as general in application as any method involving the use of aqueous media, although the reviewer has not yet had the opportunity to demonstrate the truth of this statement. However Schneider and Peterman were able to use their original procedure[14] to obtain nuclei from spleen and certain tumors, as well as from liver. Here, a high-speed mixer was used to break the cells.

In general it is very difficult to obtain clean nuclei from tumor cells by procedures involving the use of aqueous media except by the use of strong citric acid (2 to 5%) (see footnote [9]). A certain proportion of the cells usually resists breaking and subsequently causes heavy contamination of the nuclear fraction. Even when reagents such as "hexameta" phosphate (a decalcifying material) or thioglycolic acid are used, many unbroken cells are usually found.

It has been found that if the method with very dilute citric acid at pH 6 is used for isolating cell nuclei from liver, the livers must be taken from fairly young animals to avoid excessive numbers of unbroken cells. This statement applies to rat, cat, rabbit, and beef liver, and is probably of quite general application. As the animal ages, more and more of the liver cells become resistant to breakage. It is impossible, for example, to achieve sufficient breakage of cells when this method is applied to cow liver.

2. Methods Involving the Use of Nonaqueous Solvents with Lyophilized Material

a. Limitations of Methods

The most obvious limitation to the use of nonaqueous procedures in isolating cell nuclei is that enzyme studies are restricted by the damaging action on certain enzymes of the lyophilization or of the solvents or both. Studies of cytochrome oxidase, for example, should for this reason be carried out on nuclei obtained in aqueous media. Mirsky *et al.*[23] state that as a general rule those enzymes which can be obtained from acetone powders

can be studied in nuclei obtained by nonaqueous procedures. However, these authors have not yet published figures showing the extent of damage, if any, to enzymes which they have studied in nuclei obtained by the nonaqueous procedure. In the reviewer's laboratory it has been found that aldolase is severely damaged in liver cell nuclei isolated by a modified Behrens technique,[6] whereas Stern and Mirsky[38] find that aldolase in wheat germ nuclei is not damaged to any extent by a similar procedure. In all studies on enzymes present in nuclei obtained by nonaqueous procedures, the extent of damage caused by the method should be ascertained and reported, or else it should be demonstrated that no such damage occurs.

A second obvious limitation to the isolation of cell nuclei in nonaqueous media is that studies of lipids cannot be made with such nuclei.

A third limitation, which is not so obvious, is that for several reasons the degree of purity of nuclei isolated in nonaqueous media is not easy to determine. In the first place, many of the nuclei become broken in the grinding procedures, and these broken pieces concentrate with the whole nuclei. Also, small pieces of cytoplasm can often be observed sticking to a given nucleus. Finally, the nuclei tend to swell and agglutinate when placed in aqueous solution prior to the application of stains for determining purity. Such agglutination makes an estimation of the amount of impurity by microscopic observation quite difficult. In the dry state or when suspended in nonaqueous solvents, the nuclei are so misshapen and angular that they are only with difficulty recognizable as nuclei.

b. Description of Apparatus for Lyophilizing Tissue

The first step in the isolation of nuclei in nonaqueous media is the lyophilization of the tissue. In this process, the temperature of the material should be kept as low as possible to insure minimal damage to the cells and minimal translocations of diffusible substrates. The manipulations, moreover, do not involve small blocks of tissue to be sectioned with the microtome, but, instead, relatively large masses of material, in the neighborhood of 50 to 200 g. or more. With the type of apparatus commonly available, it is therefore necessary to lyophilize at a higher temperature than when small blocks of tissue are used. In the reviewer's laboratory a bath of ice and water has been used to insure a temperature at the worst no higher than 0°, but, since a solid block of ice is formed around the flasks during the lyophilization, the operating temperature is actually lower than this. If the material is lyophilized in the air, the temperature is also lower than 0° until the end of the procedure when the rate of evaporation becomes low. At this point the temperature rises abruptly before the tissue is quite anhydrous, and damage may result. To illustrate this point, we have found that

[38] H. Stern and A. E. Mirsky, *J. Gen. Physiol.* **36,** 181 (1952).

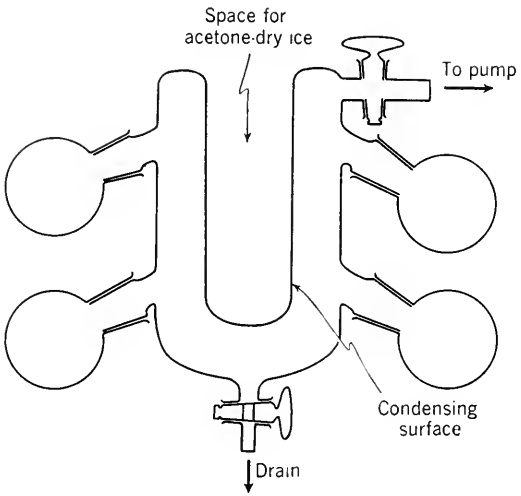

FIG. 3. Schematic drawing of one type of apparatus for lyophilizing tissue. If a large apparatus of this sort is used, the construction should be of metal, since glass may shatter. (0.2 times actual size.)

crystalline catalase suspended in water can be frozen and lyophilized without apparent damage if the flasks are surround by a bath of ice and water, whereas, if the flasks are surrounded by air, damage is caused.[39]

To cool the traps in which water is frozen out, acetone-dry ice mixtures may conveniently be used. However, if diffusible substrates are to be studied, the use of liquid nitrogen is recommended for this purpose and as low a temperature as possible should be maintained in the bath surrounding the flasks which contain the tissue. How low this temperature can be without slowing the lyophilization to a negligible speed is not known.

One type of lyophilizer which has the advantage of a very short path from the tissue to the freezing-out trap is shown in Fig. 3. This apparatus unfortunately has the disadvantage that it is difficult to surround the flasks containing the tissue with cooling baths. The type of apparatus shown in Fig. 4 has been used with considerable success in the reviewer's laboratory. It is included here mainly for the benefit of those who are not expert in freeze-dry techniques. The path between the material being dried and the first flask is kept as wide as possible. (Note especially the large-bore stopcock.) Ordinary Dewar flasks are used to hold the mixture of acetone and dry ice which is used for cooling, but a metal container should be used to hold the ice and water surrounding the centrifuge bottles, since a Dewar flask may explode if a solid block of ice is formed in it during the course of the lyophilization. It is desirable to tape the Dewar flasks and to place

[39] A. L. Dounce and R. R. Schwalenberg, *Science* **111**, 654 (1950).

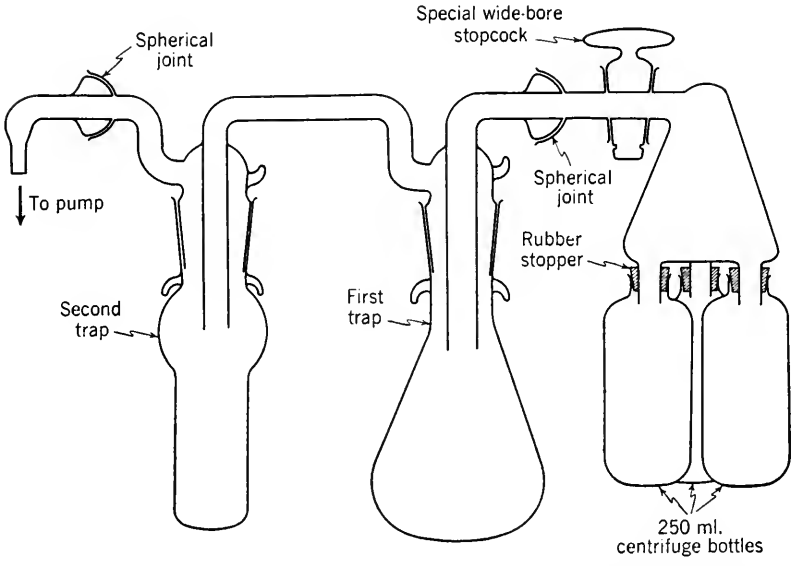

FIG. 4. Schematic construction of a second type of lyophilizing apparatus. Wide-bore tubing and a stopcock with equally large bore should be used. The two traps are cooled in Dewar flasks filled with acetone-dry ice or liquid nitrogen. The samples in the 250-ml. centrifuge bottles are cooled with ice and water in a metal container. A Dewar flask should not be used here, since it may shatter if a solid block of ice is formed around the centrifuge bottles. The Dewar flasks should be put in large tin cans and covered with heavy cloth as protection in case of accident. (0.2 times actual size.)

them in large tin cans to be covered with heavy cloth during the lyophilization.

c. Description of Methods

(1) *Behrens' Original Procedure.* In the procedure originally devised by Behrens, the removal of water was accomplished in a rather crude way by drying over a desiccant in a vacuum desiccator. The dried tissue, after being ground by a mechanical grinder and later a ball mill and then being sifted a number of times, was fractionated by specific gravity flotations in the centrifuge, with mixtures of carbon tetrachloride and benzene as suspending media. The aim was gradually to float off the whole cells and much of the cytoplasmic material, leaving the nuclei as a sediment. Subsequently the nuclei were floated off from a certain amount of heavy impurity which still fell to the bottom of the tubes in a solvent mixture of sufficiently high specific gravity to float the nuclei. Behrens and co-workers[40-44] were able to obtain nuclei from a variety of tissues, including heart muscle, brain, thymus, liver, pancreas, and wheat germ,

[40] M. Behrens, *Z. physiol. Chem.* **209,** 59 (1932).
[41] M. Behrens, *Z. physiol. Chem.* **232,** 263 (1935).
[42] M. Behrens, *Z. physiol. Chem.* **258,** 27 (1939).
[43] M. Behrens, *Z. physiol. Chem.* **220,** 97 (1933).
[44] R. Feulgen, M. Behrens, and S. Mahdihassan, *Z. physiol. Chem.* **246,** 203 (1937).

and to show the presence of one enzyme, arginase, in the liver cell nuclei. Important work on the localization of DNA in the nuclei was carried out.

(2) *Subsequent Modifications.* In subsequent modifications of the Behrens technique, one very marked improvement has been the use of modern lyophilizing techniques. Another improvement has been the substitution of cyclohexane for benzene in Mirsky's laboratory.[45] A different modification, sometimes employed, which may not be so sound on theoretical grounds, is the use of excess acetone in the cold to prepare an acetone powder from which nuclei can then be isolated by the specific gravity flotation procedure. This modification was introduced by Mayer and Gulick[46] and later by Behrens and Taubert.[47] An objection to such a procedure is that considerable translocation of material, especially that of low molecular weight, might occur before the concentration of acetone has become high enough to prevent it.

The most complete descriptions of the isolation of various types of cell nuclei by the Behrens procedure have been given by Mirsky *et al.*[45] Word for word reproductions of these descriptions would require quite considerable space, while condensations would not be of much value, and, since the material is published in a readily available journal, the reader is referred to the original article for details. Something further will be said, however, about the general principles of the method and the processing of the tissue prior to isolation of the nuclei.

Before lyophilization, the tissue, which has been frozen by means of acetone-dry ice or liquid nitrogen, is placed in a towel or other piece of cloth and is hammered into small pieces. This should, if possible, be done in the deep-freeze room to avoid local areas of wetting. A more elegant procedure for freezing, in which the tissue is ground with dry ice in a mixer, has been described by Behrens.[48] A very finely divided frozen powder is obtained by this procedure. The frozen tissue is next placed in the flasks to be attached to the lyophilizing apparatus. Centrifuge bottles are used in the apparatus shown in Fig. 4. This apparatus is of sufficient capacity to dry a relatively large amount of tissue without having to empty the traps, but care should be taken that the traps do not become plugged up. Two hundred grams of tissue, which will contain about 65 to 70% water on the average, forms a convenient amount to lyophilize.

After lyophilization, the tissue can be shredded in a Waring Blendor before being ground in the ball mill, according to Mirsky *et al.*[45] When liver tissue is used, the pieces of dry tissue may simply be crushed with a mortar and pestle and, for grinding, a commercial porcelain ball mill of 1-liter capacity with 160 irregularly shaped pebbles of 15 to 20 mm. average diameter may be used.[6] Fifty grams of the lyophilized tissue is a convenient amount to grind, although Mirsky *et al.*[45] ground 100-g. portions in a similar mill. For a 50-g. portion of tissue, 200 ml. of petroleum ether, boiling point 50 to 60°, may be added while Mirsky *et al.* use 450 ml. of the same solvent for a 100-g. portion. For a 50-g. portion of dry liver, 24 hr. is the proper length of time for grinding, while Mirsky *et al.* recommend 44 to 48 hr. for a 100-g. portion.

After the grinding operation, it is necessary to remove fiber by filtration through some sort of cloth or screen. Fine cheesecloth is very satisfactory for this purpose. The fiber remaining in the cheesecloth and the pebbles are washed with several portions of petroleum ether to avoid undue losses of nuclei. The washings must also be filtered through cheesecloth.

In principle the isolation of the nuclei from the ground, filtered tissue is simple, but in practice the procedure is laborious and time-consuming, and according to Kirk-

[45] V. G. Allfrey, H. Stern, A. E. Mirsky, and H. Saetren, *J. Gen. Physiol.* **35,** 529 (1952).
[46] D. T. Mayer and A. Gulick, *J. Biol. Chem.* **146,** 433 (1942).
[47] M. Behrens and M. Taubert, *Z. physiol. Chem.* **291,** 213 (1953).
[48] M. Behrens, *Z. physiol. Chem.* **291,** 245 (1953).

ham and Thomas[49] somewhat variable from preparation to preparation. The writer agrees with the latter statement.

The first step in the separation is to remove as large a mass of cytoplasmic material as possible without losing any appreciable quantities of nuclei. For this purpose the ground tissue is suspended in a solvent mixture of rather low specific gravity compared to that of the nuclei, such as 50% benzene, 50% carbon tetrachloride by volume (Dounce *et al.*[6]), or 40% cyclohexane, 60% carbon tetrachloride (Mirsky *et al.*[45]). An opaque supernatant without too much crust should be obtained in this step, and, if this is not found, the specific gravity of the suspending fluid must be changed slightly. The supernatant is discarded, and the sediment can be subjected to the same treatment again.

In subsequent steps the aim is to adjust the specific gravity closer and closer to that of the nuclei, while still sedimenting the nuclei, and then to raise the specific gravity to the point where the nuclei come to the top of the centrifuge tube as a mat, leaving heavy impurities in suspension and in the bottom of the centrifuge tube. It is also on occasion desirable to adjust the specific gravity of the mixture as close as possible to that of the nuclei so that the latter remain in suspension and impurities pass both to the top and to the bottom of the centrifuge tube.

In carrying out the centrifugation, it is advantageous to use relatively large volumes of the suspending medium, since entrapment of nuclei in other fractions is thereby minimized. In all steps where specific gravity flotation is being used, high centrifugal speeds (2000 to 3000 r.p.m.) are desirable, and times of centrifugation from 25 min. to 1 hr. are required, depending upon the size of the centrifuge tubes used. At the end of the preparation, it is advantageous to add a step or two of differential centrifugation in a solvent mixture of rather low specific gravity, wherein the nuclei are sedimented by slow spinning for short periods of time, leaving fine material in the supernatant.

In making up the solvent mixtures for the specific gravity flotations, the specific gravity should be measured carefully with an accurate hydrometer reading to the third decimal place. Such hydrometers should cover the range of specific gravities that are useful in the flotations (about 1.250 to 1.450). The writer and collaborators have always measured volumes and specific gravities of the solvents at room temperature, although this was unfortunately not stated in the description of the method.[6] Subsequently our fractionations were carried out at 0°, so that all specific gravities reported were presumably lower than the specific gravities obtaining during the flotation procedures in the cold centrifuge. This may account in part for failure of Mirsky *et al.*[45] to obtain good nuclei according to the reviewer's procedure, since Mirsky (private communication) measures the specific gravities after cooling the solvents. It is also implied by Mirsky *et al.*[45] that in their own procedure the specific gravity measurements were made after cooling the solvents to 2 to 4°.

According to Mirsky *et al.*,[45] the specific gravities of cell nuclei vary from tissue to tissue and within the same tissue. For chicken erythrocytes the specific gravity is said to be in the neighborhood of 1.34, and for calf thymus nuclei, about 1.37 to 1.41. This means that some heavy nuclei bearing cytoplasmic tabs will sediment together with light nuclei free from such tabs, and therefore it must be expected that in most cases the yields cannot be very high and that some fractionation of the nuclei themselves will have occurred. (The latter occurrence usually is also to be expected in methods using aqueous solvents.) Methods for determining the specific gravities of tissue components before isolation have been described by Behrens.[50]

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[49] W. R. Kirkham and L. E. Thomas, *J. Biol. Chem.* **200**, 53 (1953).

[50] M. Behrens, *Z. physiol. Chem.* **253**, 185 (1938).

A device recently reported by Behrens[47, 51] which might be very useful in the final stages of the isolation procedure is the addition of a small amount of lecithin to the suspended nuclei. This is said to prevent agglutination which occurs when the nuclei become quite free from lipid, and thus facilitates the separation of clean nuclei.

Mirsky *et al.*[45] state that they were unable to isolate satisfactory cell nuclei from normal rat liver by any procedure. Behrens stated[42] that livers of fasted animals should be used, owing to their low glycogen content. The animals chosen by him were guinea pigs and rabbits. It does not seem likely to the writer that the glycogen content in itself has much to do with ease in isolating nuclei from liver by the Behrens type of procedure. At the worst, the nuclei might be contaminated with particulate glycogen if the latter were present in high concentration. It seems likely that the specific gravity of the nuclei themselves as well as that of the cytoplasm may vary with the glycogen content of the liver, so that nuclear specific gravity might well be the determining factor in ease of obtaining a satisfactory product. Mirsky *et al.*[45] admit that nuclei of fair quality can be obtained from the livers of fasted rats. It is evident that the diet of the animal could be of importance as well as the frequency of feeding and it is likely that the large slaughterhouse animals from which organs were obtained by Mirsky *et al.* were always fasted to some degree.

In conclusion it should be stated that careful and patient work is required for the isolation of cell nuclei by any modification of the Behrens technique and that great attention must be paid to adjustments of the specific gravities of the solvent mixtures. It may be necessary to use slightly different procedures for each batch of nuclei, as can be inferred from what has been said concerning the effect of diet and fasting on the ease of production of the nuclei. The directions of Behrens *et al.* and the directions given in this Chapter for rat liver nuclei, as well as the more elaborate directions of Mirsky *et al.* for a number of tissues all can furnish guides to the proper procedure in a given instance, but the experimenter must be left to revise the details according to the particular tissue being used. It should be added that it is generally impossible to complete the isolation of nuclei in one day, but the procedure can be stopped at any point where the material is in the form of a sediment. The latter is air-dried and stored in a desiccator in the icebox or preferably the deep-freeze cabinet. When work is resumed, great care must be taken to get the dry powder completely dispersed in the solvent before proceeding with the isolation.

3. Comparison of Results Obtained Using the Various Methods Described Above, and Choice of Method for the Particular Study Being Undertaken

a. Overall Comparison of Results

Several series of results of analyses for a given substance in nuclei isolated by different procedures have now accumulated. Some of these results will be given in detail in Section II of this chapter. In some cases the results are in agreement, in others there is 100% disagreement, and occasionally there is partial agreement. For instance, the enzyme arginase has been found in liver cell nuclei prepared by a variety of methods.[1, 23, 42, 52-54] On

[51] M. Behrens and G. Seydl, *Arzneimittel-Forsch.* **1,** 228 (1951).
[52] K. Lang, G. Siebert, S. Lucius, and H. Lang, *Biochem. Z.* **321,** 538 (1951).
[53] S. Ludewig and A. Chanutin, *Arch. Biochem.* **29,** 441 (1950).
[54] A. H. Schein and E. Young, *Exptl. Cell. Research* **3,** 383 (1952).

the other hand, Lang *et al.*,[55] using methods of isolation with aqueous solvents, find a high concentration of deoxyribonuclease with a pH optimum of 5 in cell nuclei (nearly 100% of the total amount present in the cell in kidney tissue); Laskowski *et al.*[56] finds deoxyribonuclease in all cell fractions of thymus, with a lower specific activity for nuclei than for cytoplasmic particles but a firmly bound fraction in nuclei; and Schneider and Hogeboom,[57] using an aqueous medium with mouse liver, and Allfrey and Mirsky,[58] using a modified Behrens procedure, find that deoxyribonuclease is predominantly a mitochondrial enzyme. The cause of this particular discrepancy is not yet known. However, the finding of cytochrome oxidase in liver cell nuclei isolated in very dilute citric acid at pH 6 is now known to be due to the adsorption of very fine mitochondrial fragments, as has been explained, and hence other difficulties of a similar nature may be anticipated if the mitochondria are not removed while still intact. The difficulties that may arise from permeability of the nuclear membrane to enzymes has already been discussed.

If the test of gel formation with alkali or salt is used as a criterion of intactness of nuclei, it is found that nuclei isolated at pH 6 in very dilute citric acid, or, in general, nuclei isolated in sucrose solutions at pH 6.0 or higher, do not form gels and hence have suffered degradation. However, the addition of sufficient calcium chloride, as in the methods described above, results in nuclei that do form gels. Nuclei of rat liver or chicken erythrocytes isolted in 0.9% NaCl, or liver cell nuclei isolated in 70% ethylene glycol or glycerol, also will form gels. So do the Behrens-type nuclei and nuclei isolated with dilute citric acid at pH 4.0 or lower.

b. Choice of Method for Studies of Lipids

In carrying out studies of lipids, it is generally essential to obtain fairly large quantities of nuclei, and the Behrens-type procedure, for obvious reasons, cannot be employed. Hence the Waring Blendor or colloid mill should be used to obtain homogenates in aqueous media from which nuclei can be isolated. Nuclei isolated at pH 4.0 with dilute citric acid can be used to advantage, but nuclei isolated at pH 6.0 probably should not be used because of the presence of adsorbed mitochondrial frgments which may contain lipid. It seems likely that the calcium chloride-sucrose method described above would be suitable for isolating nuclei to be used in lipid studies. This method would be particularly useful if a large-scale ball-type homogenizer constructed of stainless steel could be used for homogenization.

[55] K. Lang, G. Siebert, I. Baldus, and A. Corbet, *Experientia* **6**, 59 (1950).
[56] K. D. Brown, G. Jacobs, and M. Laskowski, *J. Biol. Chem.* **194**, 445 (1952).
[57] W. C. Schneider and G. H. Hogeboom, *J. Biol. Chem.* **198**, 155 (1952).
[58] V. G. Allfrey and A. E. Mirsky, *J. Gen. Physiol.* **36**, 227 (1952).

c. Choice of Method for Study of Nucleic Acids

For work with DNA, it is very satisfactory to isolate nuclei at pH 4.0 or lower with dilute citric acid. The DNA can then be isolated by the use of detergent[59] if desired, prior to analysis. However, it should be kept in mind that severe losses of protein may occur if nuclei are isolated in aqueous media, and hence the Behrens-type nuclei would appear to be the best in studies of the *percentage* of DNA present in the cell nucleus. This statement must be accepted with some caution, however, since the purity of the Behrens-type nuclei may not be as high as that of nuclei isolated in aqueous media.

For estimating the amount of DNA per nucleus, nuclei isolated at pH 4.0[8] or lower[10-13,25] can be used, since DNA is not lost from such nuclei. Nuclei isolated at higher pH values also have been used,[6] but there probably is no advantage in this procedure.

It is not yet known what type of nuclei are best for studies of PNA. Work has been done on PNA turnover using nuclei prepared in strong citric acid, but there is a suspicion that some PNA may be lost under these conditions. Such turnover studies therefore might not reflect the turnover of total nuclear PNA.

Nuclei prepared according to Hogeboom *et al.*[22] from rat liver have a relatively high PNA content. Behrens-type nuclei may have a high[38] or a low[23] PNA content. Thus a low PNA content is not necessarily an indication of nuclear purity, and it is possible that the amount of PNA per nucleus may vary considerably. Tentatively, it is suggested that nuclei isolated near pH 4.0 with dilute citric acid may be reasonably suitable for PNA studies. The calcium chloride-sucrose nuclei isolated according to the reviewer's procedure already described might also be satisfactory if something other than gum arabic were used in the final steps of washing, or if the tissue could be perfused to render unnecessary the washing in gum arabic.

d. Choice of Method for Studies of Proteins

It is probable that no single method for isolating cell nuclei is suitable for studies of all of the protein constituents of the nuclei. If quantitative estimations of total nuclear protein are to be made, the Behrens-type of procedure is no doubt the best for isolating the nuclei, provided that some uncertainty as to the purity of the nuclei is taken into account. In the Behrens type of isolation, no protein can be lost or gained by the nuclei except possibly by diffusion during the interval between the death of the animal and the freezing of the livers, but the possibility of an appreciable exchange during this period seems remote.

Some of the nuclear globulins, at least, can be easily extracted from

[59] E. R. M. Kay, N. S. Simmons, and A. L. Dounce, *J. Am. Chem. Soc.* **74**, 1724 (1952).

Behrens-type nuclei.[6,49] Such nuclei should also be satisfactory for isolation of histone. The only possible disadvantage in the Behrens-type nuclei, apart from an uncertainty as to the purity, is that certain proteins of the nucleus may be denatured by the organic solvents used, but there is as yet little information available on this point.

Nuclei isolated in dilute citric acid at pH 4.0 seem to be satisfactory for the subsequent isolation of histone, but these nuclei are in general not satisfactory for work with proteins other than histone. They may be suitable, however, for a study of the type of protein present in the residual chromosomes described by Mirsky.

Nuclei isolated at pH 6.0 in very dilute citric acid can be used to some extent for protein studies, but it would no doubt be preferable to use nuclei obtained by one of the procedures involving calcium chloride-sucrose solutions.

It would be well however to make a comparison of the latter nuclei with the Behrens-type nuclei, since too little information is available to make possible any final judgment on the best procedure to use for isolating nuclei for the study of a given protein or protein fraction. Possible losses and gains of proteins isolated in aqueous media already have been discussed in the introduction.

e. Choice of Method for Study of Enzymes

The same remarks apply that were made concerning proteins. For the time being at least, it will be necessary in the opinion of the writer to continue work with nuclei prepared by different procedures. The Behrens type of procedure and the procedure using calcium chloride with the small homogenizer described above, are to be particularly recommended. Nuclei obtained at pH 4.0 with dilute citric acid are probably useful only occasionally in enzyme studies, and nuclei obtained at pH 6.0 suffer from the defect caused by the adsorption of fine mitochondrial fragments.

f. Choice of Method for Study of Vitamins

For studies of water-soluble vitamins and coenzymes, the Behrens-type procedure is undoubtedly the only one which would be generally reliable. For fat-soluble vitamins, nuclei obtained in dilute citric acid at pH 4.0 might be used, or nuclei isolated on a large scale using calcium chloride and the colloid mill could be tried.

g. Choice of Method for Study of Minerals

For work with minerals, the Behrens-type procedure is the only one that can be recommended at the present time. Work that has been done on the mineral content of nuclei prepared in aqueous media is probably only of slight significance.

II. Chemical Composition of the Cell Nucleus

1. Introductory Remarks

If one were to review uncritically the literature pertaining to the composition of cell nuclei, a rather impressive table of results could be compiled, some of which would however be highly contradictory and many of which would be of little or no significance. This is because many workers have given scant attention to the effects of the method of isolation of nuclei on the final composition of the purified nuclei. Thus, it is easy to find results showing the DNA concentration of mammalian liver cell nuclei to be 30% or higher, but such a high figure indicates only that much protein has been lost from the nuclei during the isolation procedure. It has been particularly difficult to arrive at a correct interpretation of the results obtained from analyzing cell nuclei for enzymes.

The following material on the composition of the cell nucleus has been assembled with due regard to probable distortions caused by the isolation procedures and accordingly some data recorded in the literature have not been included. In certain cases where it is impossible to decide which of two or more conflicting values is correct, all available data are listed together with the corresponding references.

2. Composition of the Nucleus with Respect to Lipid

a. Total Lipid

The total lipid of rat liver nuclei has been determined by several investigators. Using rats of the Osborne-Mendel strain, the writer found 3.2% total lipid in the nuclei prepared at pH 4 and 7.5% in the pH 6 nuclei.[5] Using rats of the Wistar strain, about 6.3% lipid was found in the pH 4 nuclei and about 10.8% in the pH 6 nuclei. Whether the difference between the two strains is of biochemical significance is not known, but the differences between nuclei isolated at pH 4 and pH 6 seem to be real, although of unknown cause. Possibly there is less adsorption of fine mitochondrial fragments by the pH 4 nuclei, but it is quite likely that a real variation in lipid content occurs, since the per cent DNA is about the same for nuclei isolated at pH 6.0 and pH 4.0,[31] indicating a similar protein content for both types of nuclei. In this work the lipid was extracted by means of a hot alcohol-ether mixture followed by a chloroform-methanol mixture, and all the extracted material, after evaporation of the solvents, was found to be soluble in petroleum ether. Hence, it is unlikely that differences in firmness of binding of the lipid to nuclear material influenced the results.

Total lipid has also been estimated by Williams *et al.*[60] for rat and dog

[60] H. H. Williams, M. Kaucher, A. J. Richards, and E. Z. Moyer, *J. Biol. Chem.* **160,** 227 (1945).

liver cell nuclei obtained at pH 6.0 by means of very dilute citric acid but, since the values reported for total lipid were derived by adding the values obtained for the various lipid fractions, it is likely that the values given for total lipid, which ranged from 14 to 18%, were too high. An overlapping of the various fractions is to be expected, owing to the difficulty of obtaining very pure fractions.

Values of about 11% for the total lipid of rat liver nuclei obtained by means of 5% citric acid have been reported by Levine and Chargaff.[61] The total lipid was determined by weighing and hence is presumably a true value. The fact that the values are somewhat higher than those reported by the writer is no doubt attributable to the use of strong citric acid, which extracts considerable protein from the nuclei.[9]

b. Individual Lipid Fractions

Barnum *et al.*[25] found a total phospholipid content of 3.4% in mouse liver nuclei isolated in 2% citric acid. The fractionation of nuclear lipids was first undertaken by Stoneburg[62] and subsequently by Haven and Levy[11] and Williams *et al.*[60] The results of these investigators show that nuclear lipid tends to be rich in phospholipid but is somewhat low in neutral fat. Haven and Levy found that the phospholipid of nuclei of Walker carcinoma 256 contained 60 per cent lecithin and 40 per cent cephalin, but only traces of sphingomyelin.[11] Williams *et al.*[60] on the other hand found a higher lecithin-to-cephalin ratio for dog and rat liver nuclei. Cerebroside seems to be very low or lacking in nuclear lipid.[60] Levine and Chargaff[61] found approximately a one-to-one ratio of choline to ethanolamine in lipid from rat liver nuclei, with a small amount of serine also present.

Stoneburg[62] and Levine and Chargaff[61] found that their nuclear lipid was insoluble in petroleum ether. This may have been due to the use of strong citric acid in isolating the nuclei, since the writer has found that the lipid extracted from nuclei isolated at pH 4 or pH 6 is entirely soluble in petroleum ether.

Stoneburg[62] calculated that sufficient cholesterol was present in the nuclei of muscle cells to esterify most of the fatty acids of the neutral fat fraction. This neutral fat amounted to about 40% of the total phospholipid. Williams *et al.*[60] obtained similar results. The latter investigators also found by direct experiment that most of the cholesterol of dog and rat liver nuclei was esterified, but their total cholesterol was only about 11% of the phospholipid, which is a lower percentage than can be derived from Stoneburg's data.

It is likely that the work on the lipid of isolated cell nuclei is for the most

[61] C. Levine and E. Chargaff, *Exptl. Cell Research* **3**, 154 (1952).
[62] C. A. Stoneburg, *J. Biol. Chem.* **129**, 189 (1939).

part of reasonable accuracy, since a gross transfer of lipid from cytoplasm to nuclei during the isolation is unlikely. The nuclei of the writer[8] and those of Williams *et al.*[60] which were isolated at pH 6.0 must have adsorbed some broken mitochondria, but the quantitative error thus produced may have been rather small. The nuclei isolated in strong citric acid or at pH 4 probably adsorbed less of the broken mitochondria. The nuclei prepared by Stoneburg[62] had been subjected to digestion by pepsin-HCL in addition to treatment with strong citric acid, but this should not have changed the lipid content appreciably. Further careful studies of the various nuclear lipid fractions are obviously needed.

c. Intranuclear Distribution of Lipid

Little is known of the intranuclear distribution of lipids. Isolated chromosomes contain a small amount of lipid (about 2% based on dry weight)[63] and nucleoli may contain some lipid since they tend to blacken in osmic acid. It is not yet certain whether there is generally appreciable lipid in the nuclear membrane, but Callan and Tomlin[64] believe that some is present in one of the layers of the nuclear membrane in the egg cells of certain amphibia. Since most of the nuclear lipid appears to be phospholipid, it is likely that this lipid is located in structural elements such as those just mentioned.

d. Turnover of Nuclear Lipid

Marshak,[10] Barnum and Huseby,[65] and Davidson *et al.*[66,67] have studied the turnover of nuclear phospolipid by means of radioactive phosphorus. As judged by this criterion, nuclear phospholipid has a very considerable rate of turnover, even in the resting cell, and in this respect is like all other nuclear constituents except DNA, which probably has a very low or negligible rate of turnover in the resting cell (see Chapter 26).

3. Composition of Nuclei with Respect to Nucleic Acid

a. Deoxyribonucleic Acid

It is well known that in normal somatic cells all the detectable deoxyribonucleic acid is located within the cell nucleus, and furthermore that it is very definitely a constituent of the chromosomes (see Chapter 20). Ribonucleic acid also occurs in the cell nucleus.

(1) *Percentage of DNA in Cell Nuclei.* Early work on the percentage

[63] A. Claude and J. S. Potter, *J. Exptl. Med.* **77**, 345 (1943).
[64] H. G. Callan and S. G. Tomlin, *Proc. Roy. Soc. (London)* **B137**, 367 (1950).
[65] C. P. Barnum and R. A. Huseby, *Arch. Biochem.* **27**, 7 (1950).
[66] W. M. McIndoe and J. N. Davidson, *Brit. J. Cancer* **6**, 200 (1952).
[67] R. M. S. Smellie, W. M. McIndoe, R. Logan, J. N. Davidson, and I. M. Dawson, *Biochem. J.* **54**, 280 (1953).

composition of the nucleus in regard to DNA was erroneous, largely because of failure to realize that isolation of nuclei in 5% citric acid involves removal of considerable protein with an apparent increase in the percentage of DNA, since the latter is not removed to any measurable extent. In the reviewer's laboratory it has been found as the result of much effort and several mistakes, that the DNA concentration in nuclei isolated from the livers of rats fed *ad libitum* on a fox chow diet is about 11% (based on dry weight).[4,31] This statement applies to nuclei isolated in dilute citric acid at pH 6 or pH 4, and to nuclei isolated by the calcium chloride method. Mirsky *et al.*[23] have found about 12% DNA in calf and horse liver nuclei isolated by the Behrens procedure, whereas the reviewer and collaborators found only about 4 to 5% DNA in rat liver nuclei isolated by this method. The apparently obvious inference that the latter nuclei were only about 40% pure is not necessarily well founded. Since the work of Mirsky *et al.*[23] has demonstrated that in starvation the DNA concentration in horse liver nuclei could rise to a value as high as 18%, it seems clear that diet must be taken into account in measuring DNA concentration. It has been demonstrated that *amount* of DNA in liver cell nuclei does not change materially during starvation,[68-70] although a few contrary claims can be found in the literature, such as that of Ely and Ross.[71] But nuclear protein, which is the material present in cell nuclei in highest concentration, may vary widely in starvation, so that a high concentration of nuclear DNA in starvation indicates mainly a loss in protein from the nuclei. Thus it can be seen that the *percentage* composition of the cell nucleus with respect to DNA is highly variable, even within a given organ of a single species. The percentage of DNA may also vary from one kind of nucleus to another in mammalian tissues, as can be seen from the analyses of calf thymus cell nuclei isolated by Mirsky *et al.* using a modification of the Behrens technique.[23] These nuclei contained about 26% DNA. Nuclei of tissues other than liver and thymus were found by Mirsky *et al.* to have percentages of DNA falling between the value for liver and the value for thymus.

(2) *Amount of DNA per Nucleus.* One of the most important basic discoveries concerning the DNA of cell nuclei was made by Boivin, Vendrely, and Vendrely[13,72,73] and was confirmed by Mirsky and Ris[74-76] and David-

[68] H. W. Kosterlitz, *J. Gen. Physiol.* **106,** 194 (1947).
[69] R. M. Campbell and H. W. Kosterlitz, *J. Biol. Chem.* **175,** 989 (1948).
[70] R. Y. Thomson, F. C. Heagy, W. C. Hutchison, and J. N. Davidson, *Biochem. J.* **53,** 460 (1953).
[71] J. O. Ely and M. H. Ross, *Science* **114,** 70 (1951).
[72] A. Boivin, R. Vendrely, and C. Vendrely, *Compt. rend.* **226,** 106 (1948).
[73] R. Vendrely and C. Vendrely, *Experientia* **4,** 434 (1948); **5,** 327 (1949).
[74] A. E. Mirsky and H. Ris, *Nature* **163,** 666 (1949).
[75] H. Ris and A. E. Mirsky, *J. Gen. Physiol.* **32,** 489 (1949).
[76] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **34,** 451 (1951).

son *et al.*[70,77] This is the finding that the *mean quantity of DNA per cell nucleus is constant within a given species* for normal resting diploid somatic cells and that half this value is found in spermatozoa. The amount of DNA per nucleus may, however, vary widely from one species to another.

These findings would certainly identify DNA as a chromosomal constituent, even if the chromosomal localization of DNA were not known from direct analysis and histochemical procedures. Furthermore, they lead, when taken together with work on DNA turnover, to the selection of DNA as the only known material to be a logical candidate for gene substance. Recent work by Zamenhof *et al.*[78] on one of the bacterial transforming agents (Chapter 27) greatly strengthens this hypothesis. The suggestion that genes may be composed of DNA has already been made by Mazia.[33]

The question of the amount of DNA per nucleus is discussed in detail in Chapter 19 of this book. Attention should be called to a recent paper which is concerned with errors of counting that interfere with accurate estimates of the amount of DNA per nucleus, and also with a means of overcoming these errors.[79]

(3) *State of DNA in Cell Nuclei.* The question of gel formation by isolated nuclei in relation to the state of the DNA in the nuclei has already been mentioned in this chapter and elsewhere.[4-6,80] It is the firm conviction of the reviewer that a chemical bond, of a type other than a salt linkage, normally exists between DNA and some protein of the cell nucleus, and that most of the phosphate groups of the DNA are not involved in this bond. Since it seems unlikely from the physical properties of nuclear gels that the DNA can be attached to protein by multiple bonding along the length of the nucleotide chain, the possibility of attachment of one end of the nucleic acid chain to protein must be considered. It should eventually be possible to determine the type of chemical bond in question. As long as the DNA is firmly bound to nuclear protein, the peculiar "unwinding" type of gel previously described can be formed by adding alkali, or salt at a neutral pH. When the attachment of the DNA to protein is broken, this type of gel can no longer be formed.

(4) *Intracellular Distribution of DNA.* It has been mentioned that DNA is a chromosomal constituent. Until recently it was thought that nucleoli contained PNA but not DNA but this point of view has now been questioned,[81] and will be discussed later.

[77] J. N. Davidson, I. Leslie, R. M. S. Smellie, and R. Y. Thomson, *Biochem. J.* **45,** Proc. xv (1949).

[78] S. Zamenhof, H. E. Alexander, and G. Leidy, *J. Exptl. Med.* **98,** 373 (1953).

[79] S. Albert, R. M. Johnson, and R. R. Wagshal, *Science* **117,** 551 (1953).

[80] A. L. Dounce *in* "The Enzymes" (Sumner and Myrbäck, eds.), Vol. 1, Part 1, p. 222. Academic Press, New York, 1950.

[81] M. Litt, K. J. Monty, and A. L. Dounce, *Cancer Research* **12,** Sci. Proc. 279 (1952).

b. Ribonucleic Acid (PNA)

(1) *Percentage in Cell Nuclei.* Unequivocal information is not available at present concerning the percentage of PNA in cell nuclei but it seems likely that the amount per nucleus, like the amount of protein per nucleus, will prove to vary according to the diet, tissue, species, etc.

The PNA content of liver cell nuclei obtained in aqueous solution at pH 4 or pH 6 is about 2.5%, according to the most recent work from the writer's laboratory. Previous studies indicated a content of about 5%.[31] Nuclei obtained by Hogeboom and Schneider by their most recent procedure[22] contained approximately one-quarter as much PNA as DNA; if the percentage of DNA is ten, this would correspond to rather less than 3% PNA. Rat liver cell nuclei isolated in high concentrations of acid usually contain only about one-tenth to one-eighth as much PNA,[25,82] although considerably lower amounts have been reported for other nuclei.[82]

The work of McIndoe and Davidson[66] shows that the ratio of PNA to DNA in cell nuclei can vary considerably depending on the organ and the species studied. For rat liver nuclei this ratio is in the neighborhood of 1 to 3.5; for rabbit liver, 1 to 6; for liver of tumor-bearing fowl, 1 to 5; for fowl erythrocytes, 1 to 10; and for calf thymus, 1 to 17, as calculated from a table of values showing the amounts of PNA and DNA per nucleus. Slightly different ratios are obtained from another table showing concentrations of DNA and PNA in various cell nuclei. The mammalian cell nuclei were isolated in moderately strong citric acid and the erythrocyte nuclei in buffered saline. An appreciable drop in the amount of PNA per nucleus seemed to occur in rats fasted for 72 hours.

Mauritzen *et al.*[82] found a slight increase in the ratio of PNA to total nucleic acid in nuclei of regenerating rat liver as compared with those of normal rat liver, and Leuchtenberger *et al.*[83] found a higher ratio of PNA to total nucleic acid in nuclei of certain mouse tumors than in nuclei of "normal cells."

Mirsky *et al.*[45] claim that the content of PNA in mammalian liver cell nuclei isolated by their modification of the Behrens procedure is one-tenth that of the DNA content. However, in nuclei of plant cells, Stern and Mirsky[38] find a PNA content about equal to the content of DNA.

Mirsky *et al.*[45] cite the high PNA content of liver cell nuclei obtained by Dounce *et al.*[6,31] by a modification of the Behrens technique as an indication that these nuclei were grossly contaminated with cytoplasm, but they do not mention that with one exception the figures given for PNA were derived by *calculation* from analyses for total phosphorus and direct analysis

[82] C. M. Mauritzen, A. B. Roy, and E. Stedman, *Proc. Roy. Soc.* (*London*) **B140**, 18 (1952).

[83] C. Leuchtenberger, G. Klein, and E. Klein, *Cancer Research* **12**, 480 (1952).

for DNA, and did not represent direct determinations. It has been discovered subsequently that such a procedure appears to give PNA values which are considerably higher than those obtained by direct colorimetric determination. An erroneously high value for the per cent of PNA in liver nuclei isolated at pH 4 in dilute citric acid had also been reported by Dounce *et al.*[6,31]

Until recently all direct PNA analyses done in the writer's laboratory were not based on purified PNA standards, but instead were based on a calibration curve for color production made with ATP. The technique of Schneider[84] was used with correction for color caused by DNA, and the result obtained was multiplied by two in order roughly to compensate for the fact that pyrimidine nucleosides do not give a color with the orcinol reagent, since they are not hydrolyzed. Recently it has become possible to construct calibration curves using PNA isolated from the tissues being studied by an improved procedure.[85] Thus far only nuclei isolated from rat liver at pH 4 have been analyzed but here the average value for PNA was 2.5% on a dry weight basis, a figure representing approximately one-quarter of the DNA present. This figure is now in approximate agreement with the values given by Hogeboom *et al.*,[22] McIndoe and Davidson,[66] and Mirsky *et al.*[45] for rat liver nuclei obtained by methods involving the use of aqueous and nonaqueous solvents, respectively.

Since the best sample of the Behrens-type nuclei which was used in our original work had been saved in the dry state in the refrigerator, it was also possible to reanalyze this sample directly for PNA, using liver PNA as a standard. The value obtained was approximately 3.0%.

It appears therefore that Behrens-type nuclei do possess a somewhat higher PNA content than nuclei isolated in aqueous media, and that the value for PNA is somewhat higher than reported by Mirsky *et al.*[45] for their rat liver nuclei isolated by the Behrens technique and stated by them to be impure. A comparison of different samples of nuclei on the bases of DNA content would however be much more reliable.

A few final words of caution should be added concerning estimations of the PNA content of cell nuclei. In the first place, methods for determining PNA may not be entirely precise even at the present time, and possibly more than one procedure should be used in making the analysis. But more important than this is the likelihood that the PNA content of cell nuclei may vary with a number of factors, as does the protein content. A substance with a very high rate of turnover, such as is shown by nuclear PNA,[65,67,86-89] can hardly be expected to remain in fixed amount in the nucleus, since

[84] W. C. Schneider, *J. Biol. Chem.* **161,** 293 (1945).
[85] E. R. M. Kay and A. L. Dounce, *J. Am. Chem. Soc.* **75,** 4041 (1953).
[86] A. Marshak and F. Calvet, *J. Cellular Comp. Physiol.* **34,** 451 (1949).

it is unlikely that the rate of degradation could always exactly match the rate of synthesis. Moreover, it has been pointed out that the PNA content of different types of nuclei can differ greatly, and that starvation may tend to lower the quantity of PNA in the nuclei of liver cells, at least, while increased rate of mitosis may tend to elevate it. Therefore, in future studies of the PNA content of cell nuclei, account should be taken of species, type of tissue studied, rate of cell division, diet of the animal, etc., in order to avoid the mistake of attempting to generalize from insufficient data.

(2) *Intranuclear Distribution of PNA.* A small amount of PNA is found in isolated chromosomes,[90] and, since this remains firmly bound in the residual chromosomes after removal of the DNA and histone, it seems likely that PNA is a true chromosomal constituent. The results of numerous histochemical studies have indicated that there is a relatively high concentration of PNA in the nucleolus. However certain instances of Feulgen-positive liver nucleoli have been reported,[91] and it is very common to observe a heavy ring of Feulgen-positive material around the nucleoli. Nucleoli isolated from starfish eggs[18] have been reported to contain a small amount of PNA but no DNA, whereas nucleoli isolated from liver cell nuclei were found to contain much DNA and only a few per cent PNA.[81] Hence it can be stated that PNA is in all probability very generally present in nucleoli, but it is unsound to conclude, as some have done, that all of the nuclear PNA is in the nucleolus. Actually, it is likely from consideration of the ratio of nucleolar volume to nuclear volume that a very small percentage of the total nuclear PNA is located as a rule in the nucleolus. The nuclear sap of amphibian oocytes seems not to contain PNA.[92] It is not known whether the nuclear sap of somatic cell nuclei contains PNA.

(3) *Turnover Studies as Applied to PNA.* As previously mentioned, it has been found that the PNA of cell nuclei apparently has a considerably higher rate of turnover than the PNA of any of the cytoplasmic particles, as judged by the rate of incorporation of radioactive phosphorus.[65,67,86-89] This finding, taken together with the observed diminution in the amount of cytoplasmic PNA in the denucleated amoeba,[93,94] suggests the possibility that nuclear PNA may be a precursor for at least part of the cytoplasmic PNA. This question is discussed in Chapters 26 and 28. However, since there appears to be a chemical difference between nuclear and cyto-

[87] R. Jeener and D. Szafarz, *Arch. Biochem.* **26,** 54 (1950).
[88] R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.* **195,** 257 (1952).
[89] R. M. S. Smellie and W. M. McIndoe, *Biochem. J.* **52,** Proc.xxxii (1952).
[90] A. E. Mirsky, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 143 (1947).
[91] J. N. Davidson and C. H. Waymouth, *J. Physiol.* **105,** 191 (1946).
[92] G. L. Brown, H. G. Callan, and G. Leaf, *Nature* **165,** 600 (1950).
[93] D. Mazia and H. L. Hirschfield, *Science* **112,** 297 (1950).
[94] J. Brachet, *Experientia* **6,** 294 (1950).

plasmic PNA,[95-97] independent synthesis in the cytoplasm of some of the cytoplasmic PNA is to be suspected. Such a situation in which cytoplasmic PNA would be partly dependent and partly independent of nuclear PNA would fit well with the concept of plasmagenes expressed by Spiegelman and Kamen.[98]

4. Composition of the Cell Nucleus with Respect to Proteins

The principal known protein fractions of the cell nucleus are the histone fraction, a globulin fraction, a newly discovered acid protein,[99-101] and residual insoluble protein. The acid protein, however, may be identical with the protein of the residual chromosomes of Mirsky.[90] It is not clear whether cell nuclei contain appreciable quantities of albumins, but at least small amounts may be present. It is very likely that albumins, if present, will be lost, together with some of the globulin fraction, from nuclei isolated in aqueous media.

a. Scheme for Separating Nuclear Protein Fractions. The following scheme can be used to fractionate the major proteins of cell nuclei: (a) Extract the nuclei, previously isolated so as to keep the DNA firmly bound, twice with 0.9% NaCl solution at pH 7.0, and wash twice with water. The globulin fraction together with any albumins that may be present will pass into solution. (b) Extract the residue twice with 0.2 *N* HCl and wash twice with water. (A quantity of HCl equivalent to five times the volume of the packed nuclei suffices for each extraction.) The histones will be extracted. (c) The residue contains DNA, presumably PNA, and the lipoprotein of Mayer *et al.*[100] and Laskowski and Engebring,[99, 101] which may be identical with the residual chromosomal protein of Mirsky[90] and with the "chromosomin" of the Stedmans.[101a] It is apparently possible to obtain the lipoprotein in solution by treatment of the residue with deoxyribonuclease, followed by extractions with alkali according to Laskowski.[99, 101] It should be noted that the residue forms the "unwinding" type of gel typical of nuclei containing firmly bound DNA. The DNA thus is probably bound firmly to the residual protein or liproprotein of cell nuclei rather than to the extractable histone. The method of Mayer *et al.*[100] for isolating the acid lipoproteins seems to be applicable only to nuclei that do not have the DNA firmly bound.

All operations should be carried out at a temperature as close to 0° as possible. Globulins can be recovered from the NaCl extract by dialysis at pH 5 to 6, and histones can be recovered from the HCl extract by the addition of ammonia which causes them to precipitate. If insufficient ammonia is added, only part of the histone will precipitate. One part of concentrated ammonia by volume to ten parts of his-

[95] D. Elson and E. Chargaff, *Federation Proc.* **10,** 180 (1951).
[96] A. Marshak, *J. Biol. Chem.* **189,** 607 (1951).
[97] G. W. Crosbie, R. M. S. Smellie, and J. N. Davidson, *Biochem. J.* **54,** 287 (1953).
[98] K. Spiegelman and M. D. Kamen, *Science* **104,** 581 (1946).
[99] M. Laskowski and V. K. Engebring, *Federation Proc.* **11,** 246 (1952).
[100] T. Y. Wang, D. T. Mayer, and L. Thomas, *Exptl. Cell Research* **4,** 102 (1953).
[101] V. K. Engebring and M. Laskowski, *Biochim. et Biophys. Acta* **11,** 244 (1953).
[101a] E. Stedman and E. Stedman, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 224 (1947).

tone solution will suffice if the histone solution is not excessively dilute. A second histone fraction, soluble in ammoniacal solution, can be precipitated by adding five volumes of alcohol.

b. Amount of Total Nuclear Protein. The amount of protein in cell nuclei varies with the type of nucleus. In rat liver cell nuclei isolated from rats fed *ad libitum* on a fox chow diet, the amount of total protein is about 80 to 85%, if the nuclei are isolated in aqueous media. In Behrens-type nuclei isolated in the writer's laboratory, it can be calculated from the DNA content that the amount of total protein would be close to 90% on a dry weight basis. The ratios of protein to DNA in the Behrens-type nuclei isolated from cow and horse liver are 5.7 and 7.3, respectively, from which it can be calculated that the corresponding percentages of protein are 85 and 88%. If an allowance for lipid is made, the percentage of protein in both cases would be lowered slightly. On the other hand, the protein content of calf thymus cell nuclei is only 74% (without allowance for lipid), and the protein content of the nuclei of spermatozoa may be considerably lower than the latter figure, although this point is not well established since the nuclei of sperm cells have not been isolated by the Behrens procedure. In any case, it seems clear that the protein content of cell nuclei is far higher than was thought by the early workers, and that protein is the principal chemical constituent of cell nuclei, at least in terms of the *amount* present.

c. Albumin Content. The percentage of albumin in cell nuclei is not known, but it cannot be high.

d. Globulin Content. According to Kirkham and Thomas,[49] the globulin content of calf thymus nuclei is about 26%, and that of calf liver about 42%. In this case, the nuclei studied were isolated by a modification of the Behrens technique. The difference in the globulin content of the two types of nuclei in question is reflected in a difference in the DNA content. Mirsky *et al.*,[45] also using nuclei isolated by a modification of the Behrens technique, found that calf thymus nuclei contained 26% DNA and calf liver nuclei 15% DNA (see under *Percentage of DNA in Cell Nuclei*, p. 122). Since the DNA per resting somatic cell nucleus is constant in the calf (neglecting polyploidy), the high percentage of globulin in the liver nuclei apparently causes the percentage of DNA to be low. It should be noticed that any albumin present in the nuclei studied by Kirkham and Thomas[49] would have been estimated as globulin.

e. Histone Content. The histone fraction of cell nuclei may not be a homogeneous protein.[29,101,102] The amino acid composition of histone has been studied by Davidson and Laurie,[103] Hamer,[104-106] and Mirsky *et al.*[107]

[102] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **31,** 7 (1947).
[103] J. N. Davidson and R. A. Laurie, *Biochem. J.* **43,** Proc. xxix (1948).
[104] D. Hamer, *Nature* **167,** 40 (1951).
[105] D. Hamer, *Brit. J. Cancer* **5,** 130 (1951).

An extensive study of the histone content of whole cell nuclei has recently been made by Stedman and Stedman.[107a] Their data show values for histone sulfate ranging from 17 to slightly more than 30% for various types of cell nuclei isolated in 4% acetic acid solution. These results may be valid for the type of preparation in question, but no allowance has been made for a possible partial loss of histone and a certain loss of much nonhistone protein during the isolation procedure, since the Stedmans do not believe that any such loss occurs. Nevertheless, the high DNA content of their cell nuclei constitutes direct evidence of loss of much protein. Unfortunately many of the conclusions drawn by the Stedmans from their experimental data, however excellent the latter may be, are, in the opinion of the writer, highly questionable.

It has been demonstrated[9] that an increasing loss of protein from nuclei occurs as the pH is lowered below 4.0. It was thought at one time that the extracted protein was mainly histone, but this is not necessarily true, although it is likely that at low pH values at least some histone is lost. Therefore, it is difficult or impossible to apply a correction factor to the Stedmans' results to allow for the probable effect of strong acid in reducing the percentage of histone in the nuclei. Recent analyses of rat liver cell nuclei isolated at pH 4.0 in the writer's laboratory have shown a histone content of about 17 to 20%.

f. Protamine Content of Nuclei of Spermatozoa. The basic protein of fish sperm nuclei is protamine. This low-molecular-weight basic protein, which unlike histone can be slowly dialyzed through a collodion membrane,[108] comprises a large fraction of the nucleus, but just how large is still uncertain. The older workers thought that nucleoprotamine comprised about 81% of the lipid-free sperm cell nucleus. Mirsky and Pollister[108] found an even higher amount (about 91%) but called attention to the probable importance of the remaining nonprotamine protein. From data in this reference[108] and from that in an earlier paper of Mirsky and Pollister,[109] it can be calculated that trout sperm nuclei contain around 63% protamine. Owing to the fact that spermatozoa nuclei have been isolated only in aqueous media, values of the content of various protein fractions are not known with very great certainty.

g. Content of Acidic Lipoprotein (Residual Protein). According to Mayer *et al.*,[100] the liproprotein fraction of cell nuclei isolated from rat liver in very dilute citric acid at pH 6.0 amounts to about 50% by dry weight of these

[106] D. Hamer, *Brit. J. Cancer* **7**, 151 (1953).

[107] M. M. Daly, A. E. Mirsky, and H. Ris, *J. Gen. Physiol.* **34**, 439 (1951).

[107a] E. Stedman and E. Stedman, *Phil. Trans. Roy. Soc. (London)* **B235**, 565 (1951).

[108] A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.* **30**, 101 (1946).

[109] A. E. Mirsky and A. W. Pollister, *Proc. Natl. Acad. Sci. U.S.* **28**, 344 (1942).

nuclei. This result is reasonable, since the sum of the DNA, PNA, histone, and globulin could easily add up to 40% or more. Judging from the method of isolation of the lipoprotein and the total amount present, it would appear that the protein component of this material is very probably the same as the protein material found in the residual chromosomes of Mirsky.[90]

Mayer *et al.*[100] have isolated the lipoprotein fraction in question by exhaustively extracting the nuclei with 1 *M* sodium chloride, and then dissolving the lipoprotein in 0.1 *N* alkali. The lipoprotein is subsequently recovered by precipitation through lowering of the pH to 5.7 to 6.0. The NaCl extraction removes DNA as well as globulin from the nuclei, since the DNA is not firmly bound in the pH 6 nuclei. The method presumably would fail if applied to nuclei isolated at pH 4 or lower, since in such nuclei the DNA is firmly bound, apparently to the residual protein, and will not dissolve in 1 *M* NaCl. However, the method of Laskowski using deoxyribonuclease[101] should be applicable in this case.

h. Turnover Studies. Hammarsten *et al.*[110] found a very appreciable rate of uptake of radioactive glycine by the total protein of liver cell nuclei. Miller *et al.* showed that all of the protein *fractions* of liver cell nuclei appear to show a considerable turnover, as measured by uptake of radioactive lysine,[4] although there is disagreement in regard to the histone fraction.[111] Mirsky *et al.*[112] have demonstrated a considerable rate of incorporation of radioactive glycine into the histone and "residual protein" fractions of nuclei of liver, kidney, and pancreas. The turnover values for the nuclear proteins are comparable with those for cytoplasmic proteins.[112,112a] This statement of course cannot be taken to mean that all individual proteins of the cell nucleus necessarily have an appreciable turnover in the resting cell, although there is no convincing evidence to the contrary at the present time.

The apparently negligible turnover of DNA as compared with the relatively high rate of turnover for nuclear PNA, nuclear phospholipid, and nuclear protein, is one line of evidence that has led to the choosing of DNA as the substance of which genes are probably composed. Logically it would seem that gene substance should have little or no turnover, since otherwise genic material would presumably fluctuate in concentration and might easily be lost. If a nuclear protein with no turnover in the resting state should ever be found, this might also become a possible constituent of gene material.

[110] A. Bergstrand, N. A. Eliasson, E. Hammarsten, B. Norberg, P. Reichard, and H. von Ubisch, *Cold Spring Harbor Symposia Quant. Biol.* **13,** 22 (1948).

[111] M. D. Hoberman and P. M. Peralta, *Federation Proc.* **11,** 232 (1952).

[112] M. M. Daly, V. G. Allfrey, and A. E. Mirsky, *J. Gen. Physiol.* **36,** 173 (1952).

[112a] R. M. S. Smellie, W. M. McIndoe, and J. N. Davidson, *Biochim. et Biophys. Acta* **11,** 559 (1953).

5. Composition with Respect to Enzynes

The identification of the enzymes in the cell nucleus is a field beset with many pitfalls, and in spite of the considerable amount of work which has been done in several laboratories, many of the published results are of doubtful validity. In general, too many enzymes have been studied and too little effort has been spent in attempting to determine what enzyme translocations from the nucleus to the cytoplasm and vice versa are likely to occur during the various isolation procedures. In the opinion of the writer, one of the soundest approaches to the problem of enzyme translocation available at present is to make a comparative study of nuclei isolated by different procedures. The same point of view has been expressed by Behrens.[47]

Certain results concerning the enzyme composition of cell nuclei are nevertheless fairly clear at the present time. In the following paragraphs a complete list of all nuclear enzymes studied thus far will not be given, owing to uncertainties as to the reliability of the work. A fairly complete table of enzymes of cell nuclei can be found in a recent book by Lang,[113] and a review of the present status of the enzyme chemistry of isolated cell nuclei has recently been prepared by the writer.[113a] Only a rather brief summary of results will be given here.

a. Oxidative Enzymes. In studies on the enzymes cytochrome oxidase and succinic dehydrogenase[4,80,114] in liver cell nuclei isolated at pH 6.0, it was found that although succinic dehydrogenase could scarcely be detected, cytochrome oxidase was always present at an activity per unit of dry weight of 50% or more of the corresponding activity for the whole homogenate. Schneider and Hogeboom[115] found similar results for cytochrome oxidase, but argued that the concentration observed was too low to be of significance. Succinic dehydrogenase was found by them in the same ratio to cytochrome oxidase as in the whole homogenate. The reasoning of Schneider and Hogeboom that an enzyme of a cell particulate should not be considered as of significance unless a large proportion could be recovered in the particulate in question, was criticized by Dounce[4,116] and by Mirsky *et al.*[23] It was also pointed out[4,80] that the nuclear preparations of Schneider and Hogeboom were contaminated by microscopically visible impurities to such an extent that a resolution of the disagreement could not be obtained by a study of their nuclei. Graffi and Junkman[117] had found in 1946 that nuclei isolated from mouse ascites tumor cells were practically free from cyto-

[113] K. Lang, "Der Intermediare Stoffwechsel." Springer, Berlin, 1952.
[113a] A. L. Dounce, *Intern. Rev. Cytol.* **3,** 199 (1954).
[114] A. L. Dounce, *J. Biol. Chem.* **147,** 685 (1943).
[115] W. C. Schneider and G. H. Hogeboom, *Cancer Research* **11,** 1 (1951).
[116] A. L. Dounce, *Cancer Research* **11,** 562 (1951).
[117] G. Graffi and K. Junkman, *Klin. Wochschr.* 5/6, 78 (1946).

chrome oxidase. More recently, Hogeboom and Schneider,[7,118] using an adaptation of the technique developed by R. M. Schneider and Peterman,[14] were able to isolate reasonably pure nuclei in sucrose solution, and showed that the cytochrome oxidase activity of a number of preparations of nuclei was strictly proportional to the number of mitochondria remaining with the nuclei. This work, taken together with other work just mentioned, furnished very convincing evidence of the absence of cytochrome oxidase from cell nuclei. Nevertheless it was difficult to understand why the nuclei obtained by Dounce at pH 6.0 in very dilute citric acid should have contained appreciable cytochrome oxidase, since, in spite of the implication of Schneider and Hogeboom that contaminating mictochondria must have been present, no appreciable amounts of whole mitochondria could be detected. It was eventually concluded that the cytochrome oxidase found in these nuclei must have been present as the result of adsorption of very finely divided pieces of mitochondria, too small to be noticeable by microscopic examination.[113a] This was confirmed by displacing the adsorbed mitochondrial fragments with gum arabic, and by developing the new method of isolation of nuclei using calcium chloride (described above) in which the mitochondria are very completely removed without being fragmented. It has recently been possible to obtain by this procedure nuclei which show no measureable cytochrome oxidase activity.

The complete absence of succinic dehydrogenase from the nuclei isolated by Dounce at pH 6.0 was undoubtedly due to a decay phenomenon,[113a] and not, as thought previously, to the absence of mitochondrial material.

Uricase was found by T. H. Lan[119] in high concentration in nuclei isolated at pH 6.0 from very dilute citric acid. But again this result was apparently caused by adsorption of mitochondrial fragments, since Hogeboom and Schneider[7] and Lang and Siebert,[16] using nuclei isolated in aqueous media, have found insufficient uricase to be of significance, and this result has also been obtained by Mirsky *et al.*[23] using Behrens-type nuclei.

The results with cytochrome oxidase, uricase, and succinic dehydrogenase can be considered as quite conclusive, owing to the insolubility of these enzymes under the conditions of the isolation. There can be no question of loss of the enzymes from the nuclei by extraction with the solvent, unless it is postulated that nuclear oxidases are completely different in solubility from cytoplasmic oxidases. However, it has been observed in this laboratory that the cytochrome oxidase present in liver cell nuclei isolated at pH 6 in very dilute citric acid solution appears to be just as insoluble as the mitochondrial cytochrome oxidase.

D-Amino oxidase also is probably lacking in cell nuclei, in spite of the

[118] G. H. Hogeboom, W. C. Schneider, and M. J. Striebich, *Cancer Research* **13**, 617 (1953).

[119] T. H. Lan, *J. Biol. Chem.* **151**, 171 (1943).

fact that the enzyme was found by Lan[119] in high concentrations in liver cell nuclei isolated at pH 6.0 in very dilute citric acid. Again, D-amino oxidase is an insoluble mitochondrial enzyme and thus will undoubtedly be present in the fine mitochondrial fragments produced by the action of the Waring Blendor when it is run rapidly enough to disrupt a high proportion of liver cells. No D-amino oxidase was found in cell nuclei isolated by Lang and Siebert.[120]

Before consideration is given to other classes of nuclear enzymes, most of which are water-soluble, the reader should be reminded that what has been said concerning methods of estimating soluble proteins of the cell nucleus applies for the most part to determinations of nuclear enzymes. It is still a matter of dispute as to whether the nuclear membrane is permeable to water-soluble proteins or not, but Anderson[121-123] has assembled a fair amount of evidence in favor of permeability, against which there is only rather meager evidence to the contrary.[4,7,113a] In any case, it can be shown by direct experiment that under experimental conditions frequently employed in isolating nuclei, protein can be extracted.[6,31,45] Accordingly, one must always anticipate the possible loss of a given soluble nuclear enzyme during the isolation procedure. Such a loss for instance has apparently been directly demonstrated by Mirsky for nucleoside phosphorylase.[45] The use of a high pH or a very low pH or saline in the homogenizing medium can be expected to produce a particularly severe loss in soluble nuclear enzymes.[31]

Judging from work with arginase,[1] soluble enzymes are not necessarily adsorbed to an appreciable extent from the aqueous homogenate by the nuclei. Such adsorption of adenosinetriphosphatase by nuclei may occur, according to results obtained by Mirsky *et al.*,[23] although this might possibly be another instance of adsorption of finely divided mitochondrial fragments carrying the adenosinetriphosphatase rather than an adsorption of a soluble enzyme.

In general, different methods for isolating cell nuclei in aqueous media seem to lead to more concordant results for water-soluble, than for water-insoluble, enzymes, although even here not all results are in agreement.

Owing to the possibility of loss of a given enzyme during the extraction procedure, on the one hand, or adsorption of enzymes by the nuclei, on the other hand, Mirsky *et al.*[23] maintain that the Behrens type of procedure should always be used for a study of nuclear enzymes when this type of isolation does not cause serious damage to the enzymes being studied. Unfortunately, these authors do not list the extent of damage to the enzymes studied by them, although, owing to possible differences in degree of

[120] K. Lang and G. Siebert, *Biochem. Z.* **320,** 402 (1950).
[121] N. G. Anderson, *Science* **117,** 517 (1953).
[122] N. G. Anderson, *Exptl. Cell. Research* **4,** 306 (1953).
[123] N. G. Anderson, *J. Tenn. Acad. Sci.* **27,** 198 (1952).

damage to a given enzyme depending upon its location in the cell, such data would be very desirable. The writer is inclined not to go so far as Mirsky *et al.* in condemning procedures for isolating nuclei in aqueous media, and believes that even in the case of soluble enzymes there is still room for considerable work of a comparative nature, utilizing different methods of isolation, both aqueous and nonaqueous. This belief is founded in part on an uncertainty as to the purity of the Behrens-type nuclei, and in part on the possibility of unequal damage to an enzyme in nuclei and cytoplasm which might be caused by the Behrens technique. In addition, it has not yet been demonstrated that the Behrens-type procedure is always free from artifacts caused by adsorption, or some other type of enzyme translocation.

Although only two of the enzymes of the glycolytic system can be termed oxidative enzymes, the glycolytic system as a whole will be considered at this point for convenience, and certain of the individual enzymes of glycolysis will then be discussed. The glycolytic enzymes can all be brought into solution in aqueous media without difficulty, and they are apparently lacking in mitochondria, so that, even if nuclei are contaminated by adsorption of mitochondrial fragments, these fragments cannot be expected to cause misleading results in analyses for glycolytic enzymes.

G. T. Beyer and Dounce found in nuclei isolated in very dilute citric acid at pH 6[27,124,125] reasonably high concentrations (about half those for whole homogenate) of several glycolytic enzymes, viz., aldolase, 3-phosphoglyceraldehyde dehydogenase, enolase, and lactic dehydrogenase. Phosphorylase activity was also demonstratable after breaking the nuclei by grinding in sand. In demonstrating lactic dehydrogenase by the Thunberg technique with methylene blue, it was necessary to add diaphorase, since this enzyme (or more properly diphosphopyridine nucleotide-cytochrome C reductase) was not present in the nuclei. The necessity for adding diaphorase has been overlooked in earlier work.

Lang and Siebert[16,126] claim to have demonstrated weak anerobic glycolysis in their isolated nuclei starting from fructose diphosphate, but have concluded that the degree of glycolysis is probably too low to be of significance from the standpoint of nuclear metabolism. However, in view of the probable loss of intermediate substrates, cofactors, and to some extent the apo-enzymes themselves, this work cannot be considered to be of more than qualitative significance.

Stern and Mirsky,[127] studying plant cell nuclei, have found several glycolytic enzymes in reasonably high concentrations, and have concluded that

[124] A. L. Dounce and G. T. Beyer, *J. Biol. Chem.* **173,** 159 (1948).
[125] A. L. Dounce, S. R. Barnett, and G. T. Beyer, *J. Biol. Chem.* **185,** 769 (1950).
[126] K. Lang and G. Siebert, *Biochem. Z.* **322,** 196 (1951).
[127] H. Stern and A. E. Mirsky, *J. Gen. Physiol.* **36,** 181 (1952).

part of the glycolytic system is probably an essential component of cell nuclei. Their evidence that glycolysis does not proceed all the way to lactic acid, but instead stops at pyruvate, is however not convincing. Apparently hexokinase is not present in cell nuclei,[126] so that fructose diphosphate must be used as substrate. More work on this point is desirable.

Glycolytic enzymes have thus been found in cell nuclei isolated by three different procedures, and it is reasonable to suppose that these enzymes may be true nuclear constituents. Since in most cells a certain amount of metabolic screening of oxygen from the nucleus by the mitochondria can be expected, it may be that the glycolytic system is of importance in generating energy for metabolic processes occurring within the nucleus. The opinion of Lang *et al.*[126] that the nucleus derives energy from the hydrolysis of ATP by adenosinetriphosphatase seems unfounded, although it is possible that the nucleus might import ATP from the cytoplasm, to be used subsequently in furnishing energy for synthetic reactions through phosphate transfer.

Certain miscellaneous oxidative enzymes have also been investigated in regard to their presence or absence in cell nuclei. Catalase probably occurs in liver cell nuclei[6,23,80] but may not always be present in appreciable concentration in nuclei.[23]

Choline oxidase was absent from liver cell nuclei isolated by Lan[119] at pH 6.0 in very dilute citric acid. However, the whole hydrogen transport system and cytochrome oxidase would have been needed to demonstrate the enzyme by the technique which he used. Christie and Judah[128] found that two components of choline oxidase of rat liver were predominantly mitochondrial enzymes. Xanthine oxidase was shown to be absent from cell nuclei isolated in strong sucrose solution by Lang *et al.*,[120] but this is a water-soluble enzyme and the work should be repeated if possible with nuclei isolated by the Behrens technique.

Malic dehydrogenase was found by Dounce[80] in liver cell nuclei isolated at pH 6.0 in very dilute citric acid, but in relatively low concentration. This enzyme apparently occurs in mitochondria as well as in soluble supernatant fractions, and it remains in some doubt whether it is a true nuclear enzyme or not.

In summary, it can be said that cell nuclei are almost certainly lacking in a number of important oxidizing enzymes which are primarily mitochondrial constituents (cytochrome oxidase, succinic dehydrogenase, uricase, and D-amino oxidase) and are probably lacking in others (choline oxidase and xanthine oxidase). The complete Krebs cycle certainly cannot function in cell nuclei, owing to the lack of succinic dehydrogenase and probably of other enzymes of the cycle. However, the glycolytic enzymes

[128] G. S. Christie and J. D. Judah, *Proc. Roy. Soc.* (*London*) **B141,** 420 (1953).

may constitute a real and important nuclear enzyme system, although there is room for considerably more work on this subject. Hexokinase seems to be absent from cell nuclei.

b. Hydrolases. A number of hydrolytic enzymes have been reported in cell nuclei. *Arginase* was the first enzyme to be found in isolated cell nuclei (Behrens,[42]). Arginase has also been found in high concentration in rat liver cell nuclei isolated at pH 6 in very dilute citric acid[1] or by a modification of the Behrens technique.[6] This enzyme has also been found in mammalian cell nuclei by Mirsky *et al.*,[23] who also used a modification of the Behrens technique. The work of Dounce and G. T. Beyer[1] indicated that dissolved arginase is not adsorbed to a sufficient degree by liver cell nuclei to account for their high arginase activity.

Although arginase is almost certainly a constituent of mammalian liver cell nuclei, it is very scarce in or absent from isolated mammalian kidney cell nuclei[1,23] and chicken liver nuclei, so that it is probably not generally a nuclear constituent.

Adenosinetriphosphatase is another hydrolytic enzyme thought by some to be an important nuclear constituent, but this point is now in doubt. Nuclear sediments obtained in sucrose (which were not of a high state of purity) showed high adenosinetriphosphatase activity,[115] as also did purified nuclei obtained by Lang *et al.*,[126] who used strong sucrose solution as the medium of isolation. Nuclei isolated at pH 6 in very dilute citric acid show high adenosinetriphosphatase activity,[128,129] as also do those isolated by the new method already described, involving the use of the ball-type homogenizer and calcium chloride–sucrose solutions followed by adjustment to pH 6.2 with citric acid.[130] However, Mirsky *et al.*[23,46] found little adenosinetriphosphatase in nuclei isolated from various tissue by their modification of the Behrens technique.

In this case, it seems unlikely that contamination by adsorbed mitochondrial fragments could be causing the adenosinetriphosphatase activity of the nuclei isolated in aqueous media, since use of the ball-type homogenizer should effectively eliminate this source of error, and the situation may be cited as an excellent illustration of the necessity for comparative studies using nuclei isolated by different procedures. The nuclear adenosinetriphosphatase does not seem to be activated by dinitrophenol and might conceivably be different from mitochondrial adenosinetriphosphatase. It is suggested that no definite conclusions be drawn concerning the presence of adenosinetriphosphatase in isolated nuclei until further work has been done.

Deoxyribonuclease is an interesting hydrolase concerning the intracellular localization of which there is considerable disagreement. According to

[129] H. A. Lardy and Harlene Wellman, *J. Biol. Chem.* **201**, 357 (1953).

Laskowski *et al.*[56] and Lang *et al.*[55] the enzyme is highly concentrated in cell nuclei (thymus and kidney) while according to Webb[131], Schneider and Hogeboom,[57] and Mirsky *et al.*[58] it is essentially mitochondrial. Clearly in this case also, more work with nuclei isolated by different methods will be required to settle the matter.

Alkaline phosphatase is an enzyme the intracellular distribution of which has been studied extensively by cyto- and histochemical techniques. It was originally taken for granted that this enzyme was present in high concentration in cell nuclei, largely as the result of histochemical studies (see footnote 80), but also because isolated chromosomes[90] and isolated nuclei generally showed high enzyme activity. Subsequently, it was demonstrated that diffusion of the newly formed calcium phosphate, before its precipitation, could cause artifacts in the histochemical method,[132,133] and doubts arose as to whether the apparent nuclear localization of the enzyme was valid. These doubts were strengthened by the observation that a different histochemical procedure showed a lack of alkaline phosphatase in liver cell nuclei.[133]

Nuclei isolated in dilute citric acid from calf liver cells show a high alkaline phosphatase activity relative to that of the whole homogenate, but we now realize that this could be the result of the adsorption of finely divided mitochondrial fragments, as in the case of cytochrome oxidase. However, nuclei isolated by the new technique using calcium chloride and sucrose also seem to have alkaline phosphatase in specific activity as high as that of the homogenate.[134] Mirsky *et al.*[23] found rather low concentrations of alkaline phosphatase in their Behrens-type nuclei, except in horse liver. Thus the presence or absence of alkaline phosphatase in cell nuclei is a matter requiring further study, but at the present time it can be stated that the concentration of this enzyme, in certain cell nuclei at least, is certainly lower than was indicated by the early work.

Acid phosphatase is probably not generally present in cell nuclei in high concentration, and, judging from the work of Pallade,[135] who showed it to be predominantly a mitochondrial enzyme in rat liver cells, it may actually be absent from some types of cell nuclei.

Certain peptidases and proteases have been reported as present in cell nuclei isolated in aqueous media, by Miller, Dounce, and assistants[4] and by Lang *et al.*[55] On the other hand, Maver *et al.*[136] found very low catheptic

[130] R. F. Witter, M. A. Cottone, and A. L. Dounce, unpublished.
[131] M. Webb, *Nature* **169,** 417 (1952).
[132] B. F. Martin and F. Jacoby, *J. Anat.* **83,** 351 (1949).
[133] A. B. Novikoff, *Science* **113,** 320 (1951).
[134] A. Emery and A. L. Dounce, unpublished.
[135] G. E. Palade, *Arch. Biochem.* **30,** 144 (1951).
[136] M. E. Maver, A. E. Greco, E. Løvtrup, and A. J. Dalton, *J. Natl. Cancer Inst.* **13,** 687 (1952).

activity in nuclei isolated by an improved method from normal rat liver. Nuclei of regenerating liver and normal spleen showed somewhat higher catheptic activity, amounting approximately to one-third of the specific activity of whole tissue in the case of spleen.

c. Enzymes of General Distribution in Tissues Contrasted with Enzymes Peculiar to a Particular Tissue. One of the aims in research on enzymes of cell nuclei is to find out if possible whether cell nuclei are alike or different with respect to their enzyme systems and, if they differ, whether any relationship can be found between nuclear and cytoplasmic enzymes or whether there are some enzymes common to all nuclei.

The writer very early attempted to find a relationship between the enzymes of cell nuclei and the enzymes commonly found in tumor or growing tissue[27] but was unable to do so. The work with arginase for example[1] showed that nuclei can be very diverse in regard to the enzymes which they contain. More recently, Mirsky *et al.*[23] have attempted to analyze the problem relative to particular tissues. According to Mirsky, nuclei differ among themselves as much as do the whole tissues, with respect to enzymes characteristic of particular tissues. Arginase, for instance, was in high concentration in mammalian liver cell nuclei and calf kidney nuclei relative to the concentration in whole tissue, but it was almost absent from nuclei of fowl kidney. Catalase was present in high concentration in mammalian liver cell nuclei but was absent from fowl and calf kidney nuclei. (In this laboratory catalase was found in relatively high concentration in lamb kidney nuclei isolated in dilute citric acid at pH 6.[80]) Myoglobin was not present in muscle nuclei, but hemoglobin was present in chicken erythrocyte nuclei. There was no tendency toward a high nuclear concentration of lipase, amylase, uricase, adenosinetriphosphatase, or alkaline phosphatase in the tissues studied, and in many cases the nuclear concentrations of these enzyme were particularly low.

Of enzymes of more general distribution, adenylic deaminase and nucleoside phosphorylase were always in very high concentration in the nuclei studied; esterase was often abundant; but β-glucuronidase tended to be low.

Thus no particularly simple generalizations can be derived from the work of Mirsky *et al.*[23] concerning nuclear enzymes. The subject is dealt with in greater detail in a recent review[113a] and a considerably less positive attitude towards the situation than is adopted by Mirsky may be necessary until further results are forthcoming. Certain evidence concerning the permeability of the nuclear membrane brings up the possibility that the latter may be permeable by diffusion to many enzymes, but this point of view is not accepted by Mirsky.

Hogeboom and Schneider[7] have recently reported that most of a DPN-synthesizing enzyme of liver cell nuclei is recoverable in nuclei isolated in sucrose solution by their latest procedure, in spite of the fact that this

enzyme can easily be brought into aqueous solution and therefore should be easily lost. This enzyme obviously must be one of general distribution. Hogeboom and Schneider conclude that one manner in which the nucleus might exert its effect on cytoplasm would be through formation of coenzyme I. This conception of coenzyme synthesis by nuclei, which was previously suggested by Brachet,[137] may be true, but should be regarded with considerable skepticism. In the first place, it has not been shown by Hogeboom and Schneider that DPN added to the homogenate can be quantitatively recovered. In the second place, it is not likely that mitochondria are permeable to DPN, although they contain this coenzyme. If all of the DPN of the cell is synthesized in the nucleus, it is difficult to account for intra-mitochondrial DPN. Finally, it is scarcely reasonable that genetic effects due to the nucleus could be mediated by coenzyme synthesis, and hence, even if DPN is synthesized entirely in the nucleus, this apparently can only be a very special type of nuclear function. In order that genetic effects should be passed from the nucleus to the cytoplasm, it seems necessary to conclude that large and complicated molecules, such as nucleic acid or protein or both, should likewise be able to pass from the nucleus to the cytoplasm.

The reader is referred to the references already cited for further information concerning enzymes reported to be in the cell nucleus which have not been considered here, and in particular to the table given by Lang[113] and to the papers of Mirsky *et al.*[23,58] Another analysis of the situation in regard to nuclear enzymes is given in a recent review by Hogeboom *et al.*[118]

d. Enzymes of Nucleic Acid Synthesis. The fact that nuclear PNA thus far has always shown a higher rate of incorporation of radioactive phosphate than cytoplasmic PNA indicates that enzymes involved in PNA synthesis must be present within the cell nucleus. Since DNA is a chromosomal constituent, and since the best evidence indicates that DNA is probably synthesized at the end of interphase,[70,138-141] it follows that enzymes concerned with DNA synthesis must also be present within the cell nucleus. (Claims that DNA in some cases is not synthesized at the end of interphase can be found in the literature.[142-144]

e. Enzymes of Protein Synthesis. The fact that histone and residual

[137] J. Brachet, *Nature* **168,** 205 (1951).
[138] A. W. Pollister and H. Ris, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 147 (1947).
[139] H. Swift, *Proc. Natl. Acad. Sci. U.S.* **36,** 643 (1950).
[140] H. Swift, *Physiol. Zool.* **23,** 169 (1950).
[141] P. M. B. Walker and H. B. Yates, *Proc. Roy. Soc.* (*London*) **B140,** 274 (1952).
[142] J. Pasteels and L. Lison, *Arch. biol.* (*Paris*) **62,** 1 (1950).
[143] J. Pasteels and L. Lison, *Compt. rend.* **230,** 780 (1950).
[144] J. Fautrez and N. Fautrez-Firlefyn, *Nature* **172,** 120 (1953).

chromosomal protein are, as far as is known, confined to the cell nucleus is evidence that enzymes of protein synthesis must be present within the cell nucleus.

6. Composition with Respect to Vitamins and Coenzymes

For the study of water-soluble vitamins and coenzymes, it seems certain that the Behrens-type nuclei should be used. Some work has been done by Lang *et al.*[52] on the vitamin B_{12} content of liver cell nuclei, but here nuclei isolated in aqueous media were used, and the results, showing a lower concentration of B_{12} in the nuclei than in the cytoplasm, cannot be considered as valid without confirmation by work with the Behrens-type nuclei. Williams *et al.*[145] investigated a number of vitamins in various types of cell nuclei isolated by means of the Behrens procedure, but the method of drying the tissue in the opinion of the writer was so unsatisfactory that diffusion artifacts may well have invalidated the results.

Coenzyme I is generally scarce in or absent from nuclei isolated in aqueous media, but Stern and Mirsky[127] have found this coenzyme in considerable concentration in liver cell nuclei isolated by the Behrens procedure.

For a study of fat-soluble vitamins or coenzymes, nuclei isolated in aqueous media should be used, but thus far no such studies seem to have been made. Dounce and Lan[146] found xanthophyll in nuclei of chicken erythrocytes.

7. Composition with Respect to Minerals

Dounce and Beyer[1] have published results on the metal content of nuclei isolated in dilute citric acid, and Lang *et al.*[147] have done similar work on the metal content of nuclei isolated in strong sucrose solution. The results of the studies are somewhat discordant, but in neither case can they be considered as reliable indications of the amount of metal in the nucleus as it exists within the living cell, since aqueous solvents were used in the isolation procedure. The zinc content of tumor nuclei (also isolated in aqueous media) has been studied by Heath and Liquier-Milward.[148] Poulson and Bowen[149] have reviewed the histochemical localization of various metals in cell nuclei.

The presence of calcium or magnesium or both in cell nuclei has been reported by Scott,[150] who used emission electron microscopy on sections ob-

[145] E. R. Isbell, H. K. Mitchell, A. Taylor, and R. J. Williams, *Univ. Texas Publ.* No. 4237, 81 (1942).
[146] A. L. Dounce and T. H. Lan, *Science* **97**, 584 (1943).
[147] G. Siebert, K. Lang, and H. Lang, *Biochem. Z.* **321**, 543 (1951).
[148] J. C. Heath and J. Liquier Milward, *Biochim. et Biophys. Acta* **5**, 404 (1950).
[149] D. G. Poulson and V. T. Bowen, *Exptl. Cell Research* Suppl. 2, 161 (1952).
[150] G. H. Scott, *Biol. Symposia* **10**, 277 (1948).

tained by the freeze-drying technique and subsequently subjected to microincineration.

8. Miscellaneous References

It has not been possible in the preceding two sections to cover the pertinent literature completely although every effort has been made to convey a balanced impression of concepts and investigations current when these sections were written. For the benefit of those seeking further information, a few additional references bearing on the chemistry and physiology of cell nuclei will now be given.

The following material is in part concerned with techniques of isolation of cell nuclei from various tissues: isolation of nuclei from cells of the cerebral cortex by a procedure involving the use of very dilute citric acid;[161] isolation of cell nuclei from thyroid cells by a citric acid procedure;[162] isolation of cell nuclei from normal and leukemic mouse spleen;[163, 164] isolation of muscle cell nuclei by a citric acid method;[165] and an analysis from the standpoint of morphology and histochemistry of cell nuclei isolated from mouse liver in various aqueous media.[166] The ribonuclease activity of isolated nuclei from normal and malarial parasitized chicken erythrocytes has been investigated,[167] and a new histochemical procedure for the determination of xanthine oxidase has failed to show any of this enzyme in nuclei.[168] Isolation techniques have been used in a study of the immunochemical relationship between cell nuclei, cell cytoplasm, and the products of the cell cytoplasm,[169] and in a study of the uptake of S^{35}-labeled sulfate by cell nuclei.[160] The action of anticoagulants on cell nuclei has been studied,[161-163] and work has been done on the amino acids of isolated cell nuclei.[164-166] The DNA content of sea urchin gametes has been studied,[167] and the relationship of arginine content to DNA content of erythrocyte nuclei and sperm of some species of fish has been studied.[168] Finally, further important and fascinating studies have been made of the effect of the removal of nuclei (by microdissection) from single

[161] D. Richter and R. P. Hullin, *Biochem. J.* **48,** 406 (1952).
[162] J. Rerabek, *Biochim. et Biophys. Acta* **7,** 482 (1951).
[163] M. L. Peterman and R. M. Schneider, *Cancer Research* **11,** 485 (1951).
[164] N. A. Mizen and M. L. Peterman, *Cancer Research* **12,** 727 (1952).
[165] D. S. Robinson, *Biochem. J.* **52,** 629 (1952).
[166] F. Zajdela and G. A. Morin, *Rev. hématol.* **7,** 628 (1952).
[167] Z. B. Miller and L. M. Kozloff, *J. Biol. Chem.* **170,** 105 (1947).
[168] G. H. Bourne, *Nature* **172,** 193 (1953).
[169] A. M. Schectman and T. Nishihara, *Science* **111,** 357 (1950).
[160] E. Odeblad and H. Bostrom, *Exptl. Cell Research* **4,** 482 (1953).
[161] E. Kradolfer, *Experientia* **8,** 186 (1952).
[162] N. G. Anderson and K. M. Wilbur, *J. Gen. Physiol.* **34,** 647 (1951).
[163] H. S. Roberts and N. G. Anderson, *Exptl. Cell. Research* **2,** 224 (1951).
[164] J. Blumel and H. Kirby, *Proc. Natl. Acad. Sci. U.S.* **34,** 561 (1948).
[165] B. R. Brunish, D. L. Fairley, and J. M. Luck, *Nature* **168,** 82 (1951).
[166] E. E. Polli and A. Bestetti, *Experientia* **8,** 345 (1952).
[167] D. Elson and E. Chargaff, *Experientia* **8,** 143 (1952).
[168] R. Vendrely and C. Vendrely, *Nature* **172,** 30 (1953).

cells.[169-180] Still other references are cited in reference 80 and in addition the reader is referred to two reviews.[181, 182]

III. The Nucleolus and Nucleolar PNA

1. General Remarks

Reliable biochemical information at the present time concerning the nucleolus is scanty, although there is an extensive literature concerning the cytological aspects of this cell particulate. Some of the findings are controversial, and there is even widespread disagreement as to what intranuclear inclusion bodies should be termed nucleoli. No attempt will be made here to cover work on the cytology of the nucleolus except insofar as to give the very limited amount of cytological information which is necessary for an understanding of such biochemical investigations as have been published up to the present time.

According to the views of Caspersson and Schultz,[183,184] nucleoli are supposed to be formed near *chromocenters*, which consist of *heterochromatin*, or portions of chromosomes which tend not to disperse during interphase, but rather to remain in a more or less condensed state. According to this concept, the nucleolus would seem to be structurally independent of the chromosomes but probably dependent on heterochromatin for the synthesis of some of its constituents such as PNA. Caspersson[184] has recognized difficulties in defining the nucleolus, but his own definition is not wholly satisfactory.

It is known that the eggs of amphibia and certain insects possess large numbers of nucleoli which would seem to fall into the category just described, and these nucleoli are said to migrate to the nuclear membrane and there to empty part or all of their contents through the membrane

[169] I. J. Lorch, J. F. Danielli, and S. Horstadius, *Exptl. Cell Research* **4**, 253 (1953).
[170] J. F. Horstadius, I. J. Lorch, and J. F. Danielli, *Exptl. Cell Research* **4**, 263 (1953).
[171] J. Brachet, *Symposia Soc. Exptl. Biol.* **6**, 173 (1952).
[172] N. Linet and J. Brachet, *Biochim. et Biophys. Acta* **7**, 607 (1951).
[173] F. Vanderhaeghe, *Arch. intern. physiol.* **60**, 190 (1952).
[174] M. B. Chantrenne-Van Halteren, *Arch. intern. physiol.* **60**, 187 (1952).
[175] J. Brachet, *Experientia* **8**, 347 (1952).
[176] J. Brachet, *Biochim. et Biophys. Acta* **9**, 221 (1952).
[177] E. Urbani, *Biochim. et Biophys. Acta* **9**, 108 (1952).
[178] E. Urbani, *Arch. intern. physiol.* **60**, 189 (1952).
[179] J. Brachet and H. Chantrenne, *Arch. intern. physiol.* **60**, 547 (1952).
[180] J. Brachet and H. Chantrenne, *Nature* **168**, 950 (1951).
[181] J. R. G. Bradfield, *Biol. Revs.* **25**, 113 (1950).
[182] K. I. Altman and A. L. Dounce, *Ann. Rev. Biochem.* **21**, 29 (1952).
[183] J. Schultz, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 179 (1947).
[184] T. Caspersson, "Cell Growth and Cell Function." W. W. Norton and Co., New York, 1950.

into the cytoplasm (see references cited in footnote 80). It should be noted that, since there is apparently no physical union between the amphibian egg-cell type of nucleolus and the chromosome, there is no reason to predict any simple or constant relationship between the number of such nucleoli and the number of chromosomes in the nucleus.

The egg of the common snail contains two kinds of intranuclear inclusion bodies which might be termed nucleoli but which have different staining properties.[185]

In nuclei of mammalian cells, the situation is more complex, and it is here that the greatest disputes arise as to what should be called nucleoli. No nucleolar migration has apparently been observed in mammalin somatic cell nuclei, and moreover the number of nuclear inclusion bodies that are commonly termed nucleoli by histologists and cytologists seems to depend upon the degree of polyploidy of the cell, since according to Biesele *et al.*[186] a direct relationship exists between the degree of polyploidy and number of nucleoli. Such a situation would be predicted from the cinephotographic studies of Warren Lewis on the nucleoli of rat fibroblasts.[187] Lewis found that the nuclear inclusion bodies of these cells, which are commonly termed nucleoli, are integral parts of chromosomes, and that they become dispersed during mitosis and reappear in condensed form during interphase when the chromosomes are dispersed.

Such nuclear inclusion bodies might be termed heterochromatic centers by Schultz and Caspersson,[183,184] but, if such is the case, it would appear that the "true nucleoli" of Caspersson and Schultz are generally not observed at all in mammalian somatic cells. In any event, it is such common practice to refer to the Warren Lewis type of nuclear inclusion body as nucleoli that it is very doubtful whether this trend could now be reversed even if it were desirable to do so. It might in fact be preferable to rename the inclusion bodies found in amphibian egg cells, if the question of terminology should become so important that a change should be demanded. However, it would seem simpler to retain the word *nucleolus* as a general term, and speak of two different types of nucleoli, or more than two if this becomes necessary. In this article, the general term *nucleolus* will be used to designate both the free-floating amphibian egg-cell type of intranuclear inclusion body and the intranuclear inclusion bodies of mammalian somatic cells which are attached by stalks to chromosomes.

There is one doubtful point concerning nucleoli which should be settled as quickly as possible by the biologists, namely the question of whether any of the microscopically observable intranuclear inclusion bodies found,

[185] V. Emmel, University of Rochester School of Medicine, personal communication.
[186] J. J. Biesele, H. Poyner, and T. S. Painter, *Univ. Texas Publ.* No. 4243 (1942).
[187] W. H. Lewis, *Bull. Johns Hopkins Hosp.* **66,** 60 (1940).

for example, in mammalian liver cells conform to the concept of nucleoli as enunciated by Caspersson and Schultz. It is unlikely that this is so, and at the present time it looks as though the amphibian egg-cell nucleoli may be the exception rather than the rule.

2. Electron Microscopy of Nucleoli

Nucleoli are usually so small that an intranucleolar morphology cannot easily be made out by use of the light-microscope. Moreover, the usual fixatives of histologists seem to obliterate internal structure, as can be seen by comparing electron micrographs made after using the ordinary fixatives with those made after using a superior fixative such as the buffered osmic acid of Pallade.[188]

Good electron micrographs of nucleoli within cells often show a vermiform structure.[189,190] Nucleoli in isolated nuclei, or isolated nucleoli themselves, are likely to show a vesicular structure. This may well be due to alterations of structure during the isolation procedure, although vacuolated nucleoli have also been reported by Lewis[191] in normal and malignant fibroblasts.

3. Isolation of Nucleoli

Three reports are available on the isolation of nucleoli. Krakauer[192,193] was the first to report a concentration of nucleoli from homogenates of liver cells made in very strong sucrose solutions. Photographs were not given and the degree of purity of these nucleolar fractions cannot be stated with any certainty.

Vincent reported the isolation of nucleoli from the eggs of starfish.[18] Photographs of the isolated material indicate a very high degree of purity of the material (see Fig. 5). The cells were ruptured at pH 6.0 by being passed rapidly through a No. 18 needle of a hypodermic syringe, at a temperature of 2 to 4°. The nuclei were not previously isolated, but instead the nucleoli were isolated directly from the homogenate by differential centrifugation, as in the case of the nucleolar material described by Krakauer. However, the size and morphology of the starfish egg nucleoli are such that it is unlikely that a mistake could have been caused by carrying out a direct isolation from the homogenate rather than from previously isolated nuclei.

[188] G. E. Palade, *J. Exptl. Med.* **95,** 285 (1952).
[189] E. Borysko and F. B. Bang, *Bull. Johns Hopkins Hosp.* **89,** 468 (1951).
[190] W. Bernhard, F. Haguenan, and C. Oberling, *Experientia* **8,** 58 (1952).
[191] W. H. Lewis, *Cancer Research* **3,** 531 (1943).
[192] R. Krakauer, *J. Cellular Comp. Physiol.* **39,** Suppl. 2, 66 (1952).
[193] R. Krakauer, A. M. Graff, and S. Graff, *Cancer Research* **12,** 276 (1952).

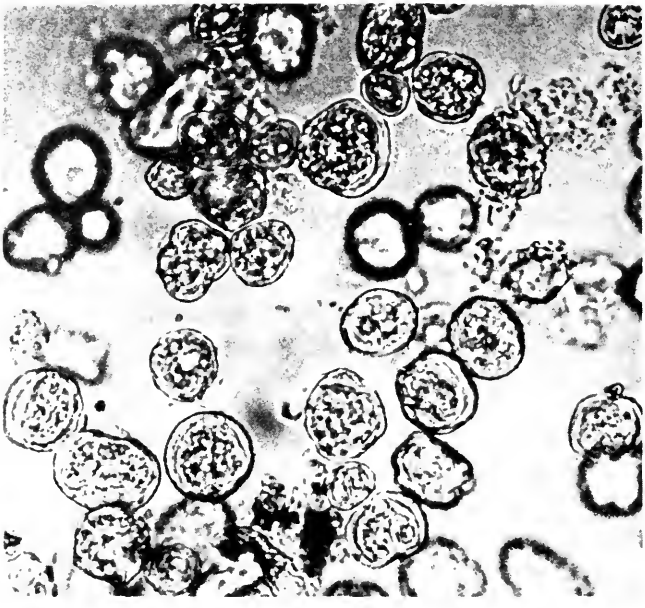

FIG. 5. Nucleoli of starfish egg cells, isolated by Vincent. (440×, unstained)

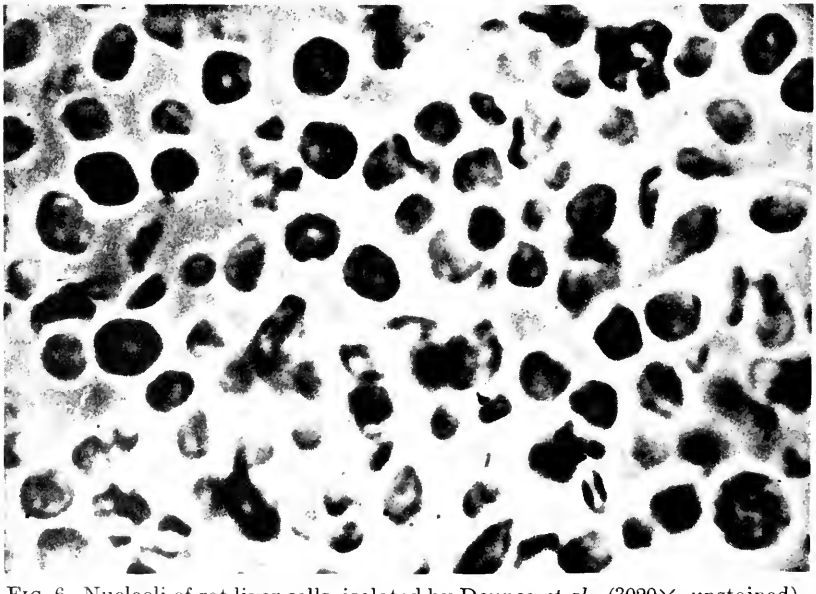

FIG. 6. Nucleoli of rat liver cells, isolated by Dounce *et al.*. (3020×, unstained)

The third isolation of nucleoli was reported by Litt, Monty, and Dounce.[81] Here the nucleoli were obtained from rat liver cell nuclei previously isolated in very dilute citric acid–gum arabic solution at pH 6.0. The nuclei were ruptured in distilled water at pH 6.2 by treatment in a 9,000-cycle magnetostriction sonic oscillator at 2 to 3° for 7 to 8 min. The nucleoli were isolated by repeated gravity sedimentations and centrifugations in distilled water and 1% gum arabic solutions. The use of already isolated nuclei as starting material precludes confusion of the nucleoli with mitochondria or other cytoplasmic particulates. The complete details of the isolation will appear elsewhere. A photograph of these isolated nucleoli is given in Fig. 6.

4. Chemical Analyses of Isolated Nucleoli

Analyses have not been reported for the nucleolar preparations of Krakauer. The starfish egg nucleoli of Vincent were chiefly protein, but contained from 2.2 to 4.6% PNA and small amounts of lipid. The PNA was qualitatively different from that of the cytoplasm in regard to nucleotide composition. No histone could be found (Caspersson considered that nucleoli contained histone). Acid phosphatase was found present, but DPN-cytochrome-c reductase, dipeptidase, and alkaline phosphatase were absent.

It is possible that these nucleoli are of the Caspersson-Schultz variety, but this point is not clear at the present time.

The nucleoli isolated by Litt, Monty, and Dounce were analyzed for DNA, PNA, and a few enzymes. The percentage of DNA was very high, ranging from 12 to 18%, while the percentage of PNA was very low—about 2 to 4% at the most. The DNA content was determined both by colorimetric and spectrographic procedures. The nucleolar concentration of DNA was higher than that of the whole homogenate of nuclei after removal from the sonic oscillator, and higher than that of either of the nonnucleolar fractions obtained therefrom.[193a] Some histone was present, as judged by the fact that 0.2 *N* HCl extracted material which was precipitable by the addition of ammonia. These nucleoli of rat liver cell nuclei thus resembled whole chromosomes in their chemical composition, a fact which is not surprising if we remember the finding of Warren Lewis that such nucleoli are indeed special parts of chromosomes.

The rat liver cell nucleoli were found to contain the enzymes aldolase, arginase, and catalase. The specific activities of arginase and catalase were considerably lower than those of the whole nuclear suspension directly after its removal from the sonic oscillator, but the specific activity of aldolase was approximately double that of the oscillated nuclear suspension, and much higher than either of the nonnucleolar fractions. Aldolase may thus be a real constituent of the nucleoli.

[193a] These fractions were the fragmented chromosomes, spun down at 18,000 r.p.m., and the opalescent supernatant from which material could not be sedimented unless the ultracentrifuge was used.

5. Histochemical Studies of Nucleoli

Almost all histochemical studies of nucleoli have led to the belief that these intranuclear inclusion bodies contain a considerable amount of PNA. A ring of Feulgen-positive material is often found around the nucleolus, however, and workers have reported Feulgen-positive nucleoli.

As an example of histochemical studies of nucleoli, some work of Pollister and Leuchtenberger may be cited.[194] These investigators have studied the nucleoli of the maize (*Zea mays*), which they term *plasmosomes*. (This term is sometimes used to designate nucleoli, apparently because the latter have staining properties more similar to those of cytoplasm than to those of other parts of the nucleus.) Nucleoli in early meiotic prophase nuclei of sporocytes were investigated, using Carnoy's acetic alcohol for fixative, and a microspectrographic technique combined with enzyme treatment was employed for analysis of constituents. Treatment of the nucleoli with ribonuclease free from deoxyribonuclease apparently caused removal of considerable material from the nucleoli, since the extinction was thereby lowered to the extent of about 53%. The statement was made that about 25% of the nucleotides (considered to be PNA nucleotides) were in the form of low-polymer material.

This work is illustrative of some of the experiments which have led most workers to the conclusion that the nucleolus contains much PNA. However, this particular investigation was concerned with plant cell nucleoli and the conclusions do not necessarily apply to liver nucleoli. The results apparently show the presence of PNA in the nucleoli, assuming that the ribonuclease was free from protease, but, since the absorption coefficient of PNA is about fifteen times greater than that of the average protein, the observed lowering of absorption of about 53% as the result of the action of ribonuclease would indicate a nucleolar PNA content of about 7% if it is assumed that no DNA was present and that protein and PNA constituted the main bulk of the nucleolar substance. However, the true PNA content might be lower than this, since the fixative may have removed constituents of low molecular weight from the nucleoli. This investigation indicates that the nucleoli of maize are largely protein, with a few per cent PNA present, and thus are similar to the starfish egg nucleoli of Vincent. In regard to protein and PNA content, the liver cell nucleoli isolated by Dounce *et al.* are also similar.

Other histochemical studies in which reliance is made on staining reactions alone are not so convincing. It is generally accepted that most nucleoli stain pink with methyl green-pyronin, but this stain does not reliably distinguish DNA from PNA but rather indicates the degree of polymerization of the nucleic acid being stained,[195-198] or possibly the way in which the

[194] A. W. Pollister and C. Leuchtenberger, *Nature* **163,** 360 (1949).
[195] C. Leuchtenberger, M. Himes, and A. W. Pollister, *Anat. Record* **105,** 107 (1949).

nucleic acid is attached to protein.[199] Depolymerized DNA will stain pink, while high-polymer PNA will stain blue or purple (see Chapter 17).

6. Conclusions

A possible error in interpreting the methyl green-pyronin staining of nucleoli has just been pointed out. Another possible error which certainly could have been made in some of the earlier histochemical studies with enzymes is the use of ribonuclease contaminated with protease or deoxyribonuclease or both, since for a time the necessity of resorting to special procedures for removing these contaminants from the ribonuclease was not realized. Another possible source of error that has been detected from work in the writer's laboratory is the failure to realize that if Feulgen staining of a particle such as a nucleolus is not exceptionally intense, this color can easily be lost in the mass of stronger color produced by the surrounding mass of nuclear material. All samples of isolated liver cell nucleoli obtained in the writer's laboratory were found to be Feulgen-positive.

What is to be concluded concerning the composition of nucleoli with respect to nucleic acid? All studies thus far published appear to indicate the presence of PNA in rather low concentration, amounting however at least to some 2 to 5% of the nucleolar mass. Protein therefore seems to constitute the bulk of the nucleolus. Considering the relatively small percentage of nuclear volume occupied by the nucleolus, which at the most cannot amount to more than a few per cent, it seems clear that the amount of PNA found in the nuclei of rat liver, for example, could not be accounted for as nucleolar PNA unless the nucleoli were composed entirely of PNA. Even then there might not be enough space in the nucleoli to contain all of the nuclear PNA. To conclude, as some have done, that all nuclear PNA is located in nucleoli would therefore be unjustified. It would likewise be unjustified to conclude that the PNA found in isolated chromosomes is confined to the nucleoli present in these preparations. It is probable that PNA is an intrinsic constituent of chromosomes as well as of nucleoli.

Concerning the presence or absence of DNA, some special comment is required. In the first place, it seems likely that many nucleoli do not contain DNA. The nucleoli of Vincent were shown by direct analysis to contain none, and the work of Pollister and Leuchtenberger cited above, and that of many other investigators, supports this statement. And yet the nucleoli isolated from liver cell nuclei in the writer's laboratory have been shown by direct analysis to contain DNA, or a very similar material. This

[196] N. B. Kurnick, *J. Natl. Cancer Inst.* **10,** 1345 (1949–50).
[197] N. B. Kurnick, *J. Gen. Physiol.* **33,** 243 (1950).
[198] N. B. Kurnick, *Exptl. Cell Research* **3,** 649 (1952).
[199] M. Alfert, *Biol. Bull.* **103,** 145 (1952).

follows from the fact that if total nucleic acid is measured (after extraction) by absorption spectroscopy, and if DNA is then measured by Schneider's adaptation of the Dische technique, the results agree within a few per cent, leaving only 2 to 4% of the material which could be PNA. Moreover, as has been stated, it has been found that isolated rat liver nucleoli are definitely Feulgen-positive. An additional observation is that some of them stain purple with the methyl green-pyronin stain, some stain pink, and some are intermediate in color, although no differences in morphology have yet been observed which might correlate with this observed variation in staining.

All these findings could be explained by assuming the presence of a rather low polymer type of DNA in the liver cell nucleoli. This assumption would explain the variable staining with methyl green-pyronin and the positive Feulgen stain, as well as the gross chemical findings. Moreover, it is possible that a low-polymer DNA might be changed by fixatives or even be partly lost during fixation. If this assumption should eventually be proved valid, it would be necessary to reinvestigate nucleoli of cells other than those of liver, to be sure that no artifacts have occurred. However, a second possible explanation of the occurrence of DNA in rat liver nucleoli is adsorption by the nucleoli of perinucleolar material rich in DNA.

This Feulgen-positive perinucleolar material which can be observed encircling the nucleoli of certain mammalian cell nuclei deserves special mention. The perinucleolar ring apparently is not an artifact of fixation as might be surmised, but is real, since Austin[200] has demonstrated that a ring showing the same morphology as the Feulgen-positive ring in rat egg cell nucleoli can be observed using the phase microscope with living egg cells. An identical ring can also be photographed with ultraviolet light. Such perinucleolar material cannot be observed in nucleoli isolated in the writer's laboratory, but it is possible that the ring might collapse on the nucleoli during the isolation procedure so as to form a thin, microscopically invisible, surface layer rich in DNA. This is a second possible explanation for the high DNA content of the isolated nucleoli.

It may therefore turn out that the Warren Lewis as well as the Caspersson-Schultz type of nucleoli do not contain DNA *within* their structure. It is suggested that the nonspecialist should keep a rather open mind on the problem of the composition of mammalian nucleoli with respect to DNA until more results have accumulated, but it may be asking too much of the specialist to suggest that he do the same.

Finally, the possible function of the nucleoli in the cell may be briefly discussed. Caspersson and co-workers[184] believe that the nucleolus is concerned with synthesis of ribonucleic acid for the cytoplasm. The observa-

[200] C. R. Austin, *Exptl. Cell. Research* **4**, 249 (1953).

tion of nucleolar migration in amphibian and insect eggs would lead to the belief that the nucleoli might transfer nucleic acid or protein or both from the nucleus to the cytoplasm. Such a transfer would make this type of nucleolus analogous to the secretory granules of cells such as those of the pancreas or salivary glands, which no doubt transfer enzymes from the cell cytoplasm through the cell wall to the ducts of the glands. It is rather obvious that the Warren Lewis-type of nucleoli cannot act in the same manner as do migrating nucleoli, and their function may well be quite different.

Yokoyama and Stowell[201] have claimed that nucleolar size in the acinar cells of the pancreas is significantly increased following pilocarpine injection. Injection of pilocarpine caused the acinar cells to secrete enzymes in the form of zymogen granules, and during regeneration of the granules the nucleolar size was said to increase. (The increase in nucleolar size as indicated by the data was however only slight, and there appeared to be a very marked decrease in size later on.) This work has led Yokoyama and Stowell to the conclusion that the nucleolus may perform some function concerned with protein synthesis in the cytoplasm.

Stowell has also observed an increase in nucleolar volume in hepatic cells following partial hepatectomy,[202] and the administration of thioacetamide is also said to cause increase in nucleolar volume.[203,204] According to Caspersson and Schultz,[205] nucleoli are large in glandular cells and in cells in which protein synthesis is active. There is a considerable literature on the nucleoli of tumor cells (see reference 186) which shows that tumor cell nucleoli are often very much enlarged, sometimes to the extent of becoming enormous relative to nuclear volume. Vacuolation also often occurs.

Thus, if an increase in nucleolar size can be taken to indicate an enhancement of nucleolar function, it would seem that mammalian cell nucleoli, which presumably are of the Warren Lewis type, have a function connected with cytoplasmic activity, possibly protein synthesis. "Proof" of nucleolar function is however unfortunately still lacking, and available evidence for concrete functions is very meager.

This brief review on nucleoli has been limited mainly to biochemical considerations, and the relatively enormous cytological literature has hardly been touched. The reader is referred to a forthcoming detailed review on the history and present status of biological concepts concerning nucleoli, which is being prepared for the *International Review of Cytology* by Dr. Vincent, the investigator previously mentioned, who isolated nucleoli from starfish eggs.

[201] H. O. Yokoyama and R. E. Stowell, *J. Natl. Cancer Inst.* **11,** 939 (1951).
[202] R. E. Stowell, *Arch. Pathol.* **46,** 164 (1948).
[203] L. J. Rather, *Bull. Johns Hopkins Hosp.* **88,** 38 (1951).
[204] A. K. Laird, *Federation Proc.* **11,** 244 (1952).
[205] T. Caspersson and J. Schultz, *Proc. Natl. Acad. Sci. U.S.* **26,** 507 (1940).

IV. Addendum

The major part of the discussion of the chemistry of cell nuclei and nucleoli in the preceding review has dealt with resting or interphase nuclei. A treatment of the chemistry of cell nuclei would hardly seem complete, however, without mention of the rather remarkable isolation of the mitotic apparatus of the sea urchin egg by Mazia and Dan,[206] although this apparatus may actually be derived from the cytoplasm rather than from the nucleus.

The method of isolation is briefly as follows. Before fertilization, the fertilization membranes of the eggs were removed by treatment with trypsin and chymotrypsin. The eggs were then fertilized and, when they had developed to the desired stage of the first cell division, they were concentrated by centrifugation and fixed in 30% ethanol at $-10°$.

A mechanical isolation of the mitotic apparatus was achieved by running the suspension of cells through a No. 25 hypodermic needle, and subsequently separating the liberated apparatus by differential centrifugation in water. This procedure, however, yielded a product somewhat contaminated with cytoplasm, and a better method was found to be treatment of the cells with H_2O_2 followed by an anionic detergent (Duponal D). In this procedure, the liberated mitotic apparatus was centrifuged down and purified by recentrifugation in water. The role of the hydrogen peroxide was to render the protein of the mitotic apparatus resistant to the action of the detergent, possibly through the formation of —S—S— linkages from —SH groups.

The isolated mitotic apparatus consisted of the mitotic spindle, the asters, and the centrosomes. In some cases chromosomes were also present, but the absorption spectrum in the ultraviolet region failed to show the presence of any nucleic acid. This is not surprising, however, since the detergent very likely rendered the DNA soluble; but it was calculated that even if no DNA had been lost, too little could have been present relative to the amount of protein to affect the absorption spectrum appreciably. The isolated mitotic apparatus appeared to consist principally of protein, which was found to be quite homogeneous, judging from its behavior in the ultracentrifuge. This protein was soluble in alkali and acid but precipitated near pH 6.

This very interesting piece of work, which has already demonstrated the chemical nature of the mitotic apparatus, may eventually prove to be of considerable importance in elucidating the mechanism of the mitotic division of cells.

Further study of nuclear enzymes isolated in sucrose and nonaqueous

[206] D. Mazia and K. Dan, *Proc. Natl. Acad. Sci. U.S.* **38**, 826 (1952).

media by Stern and Mirsky[207] has indicated that most of the protein is retained by thymus nuclei isolated in sucrose solutions, perhaps because of their high DNA content, whereas certain soluble enzymes (and hence protein) were extensively washed out of calf liver nuclei during isolation in aqueous media. The liver cell nuclei have a much lower DNA content than the thymus nuclei. A paper by Alfert and Geschwind[208] describes the use of fast green in a basic medium as a means to measure histone (or other equally basic proteins) in cell nuclei histochemically.

An inner "chromatic" nuclear membrane has been described by Brenner,[209] which is said to be composed of segments of chromosomes applied to the inside of the achromatic membrane. The book by A. Hughes[210] contains much information concerning cell nuclei.

In the writer's laboratory it has been found that 15% sucrose containing 0.005 *M* calcium chloride is an especially favorable medium for isolating nuclei from cells such as certain tumor cells which are difficult to break. The nuclei are isolated by differential centrifugation without overlaying on stronger sucrose solution. Fairly satisfactory preparations of nuclei from Walker tumor 256 have been isolated in this manner, using the new homogenizer, and this has previously been impossible without the use of strong citric acid. It has also been found that liver cell nuclei isolated in sucrose–calcium chloride solutions contain a considerably higher DNA content than nuclei isolated at pH 6.0 in sucrose solutions, and hence apparently have lost more protein than the latter, probably because of the higher pH at which the former are isolated.

In addition, it has been ascertained that the enzyme (or enzymes) responsible for loss of the ability of isolated nuclei to form gels in salt solutions or alkali lies in the mitochondrial fraction, and that this enzyme is transferred to the nuclei if the latter are isolated in such a manner that mitochondria are ruptured. If mitochondria are discarded while still intact, the enzyme is lost and the nuclei will gel. Thus nuclei isolated at pH 6.0 in sucrose solutions without the addition of calcium chloride will gel if the new homogenizer is used for rupturing the cells, so that the mitochondria are not seriously damaged. Nuclei isolated by the latest procedure of Schneider and Hogeboom, which in our experience did not form gels, probably would do so if our new homogenizer were used to break the cells.

[207] H. Stern and A. E. Mirsky, *J. Gen. Physiol.* **37**, 177 (1953).
[208] M. Alfert and I. Geschwind, *Proc. Natl. Acad. Sci. U.S.* **39**, 991 (1953).
[209] S. Brenner, *Exptl. Cell Research* **5**, 257 (1953).
[210] A. Hughes, "The Mitotic Cycle." Academic Press, New York, and Butterworth's Scientific Publications, London (1952).

CHAPTER 19

The Deoxyribonucleic Acid Content of the Nucleus

R. VENDRELY

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I. Introduction

Deoxyribonucleic acid (DNA) has been known for a long time as an essential component of the chromatin of cell nuclei. Cytochemists, using the specific Feulgen reaction, and biochemists, analyzing isolated nuclei and cytoplasmic elements, have shown that *DNA is strictly confined to the nucleus and that all nuclei contain DNA.*

In an attempt at a quantitative approach, the classical histologists described differences in stainability in nuclei from various tissues in different physiological states, but few of them used the specific Feulgen reaction. Even when they did, they could appreciate with the eye merely the density of Feulgen stain in the nucleus rather than the total amount of staining material (for the determination of which the total volume of the nucleus must be known). More recent studies by biochemists have provided a better approach to the problem of the DNA content of the nucleus. They pointed

out that the best sources of DNA are organs which are rich in nuclei[1] (thymus gland, pus cells, sperm, etc.). Examination of the tables in Chapter 16 show clearly that those tissues which contain large numbers of nuclei in a given mass are richest in DNA. There appears, therefore, to be an approximate relationship between the DNA content of a tissue and the number of its nuclei.

Techniques for the isolation of nuclei have allowed a more precise study of the question, since they have made possible the study of the chemical composition of nuclei and of the amount of DNA as a percentage of the dry weight. But the results of analyses of isolated nuclei expressed in this way do not give an exact picture of the real composition of the nuclei in living tissues since, during the isolation process, nuclei may lose part of their substance, especially protein.[2] The most satisfactory manner of expressing the DNA content of nuclei is to calculate the average amount of DNA for a single nucleus, as was done for the first time by Boivin, *et al.*[3] in 1948. The results of directed mutations in bacteria, which showed the important part played by DNA in the chemical constitution of the genes, led these authors to put forward the hypothesis that the DNA, a permanent component of the nucleus and the chief component of the hereditary material in the chromosomes, must be present in the same amount in all the cells of an animal with the exception of the haploid cells which would contain half this amount. Boivin *et al.*[3] studied the amount of DNA in individual nuclei by determining DNA in a suspension containing a known number of isolated nuclei and by calculating the average DNA content of a single nucleus. The results were in good agreement with their hypothesis. Similar results have been obtained by other authors (Mirsky and Ris,[4] Davidson *et al.*[5]).

The method of quantitative photometry in visible light (Chapter 17) allows a quantitative estimation in arbitrary units of the DNA content of one particular nucleus in a tissue section colored by the Feulgen procedure. It has been applied to this problem with results which are, in general, in good agreement with those from chemical studies.

Finally, photometry in ultraviolet light with the apparatus of Caspersson has been used to measure the DNA content of nuclei in absolute units. The problem of the DNA content of the nucleus has therefore been attacked with different tools, and, although it is a rather controversial topic at the present time, a considerable amount of data has been collected and will be discussed in this chapter.

[1] W. C. Schneider and H. L. Klug, *Cancer Research* **6,** 691 (1946).
[2] A. L. Dounce, *Exptl. Cell Research* Suppl. 2, 103 (1952).
[3] A. Boivin, R. Vendrely, and C. Vendrely, *Compt. rend.* **226,** 1061 (1948).
[4] A. E. Mirsky and H. Ris, *Nature* **163,** 666 (1949).
[5] J. N. Davidson, I. Leslie, R. M. S. Smellie, and R. Y. Thomson, *Biochem. J.* **46,** Proc. xl (1950).

II. The DNA Content of the Nucleus

1. The DNA Content of the Nucleus in the Various Tissues of the Same Animal

a. *Chemical Measurement of the DNA Content of the Nucleus in the Somatic Cells and Gametes*

Various authors studying the DNA content of isolated nuclei of animal tissues by chemical analysis have used different methods for the determination of DNA. The two most commonly employed techniques are the method of Schmidt and Thannhauser[6] and that of Schneider,[7] which have been discussed in Chapter 16. They involve the estimation of DNA either by the determination of a nonspecific element (phosphorus, purine nitrogen) or by the estimation of a specific molecular fraction, the sugar (deoxyribose).

The estimation of DNA phosphorus is convenient and is used by many authors, but the total elimination of interfering phosphorus compounds (phosphoproteins, organic phosphoric esters)[8] is essential and is not always adequately carried out. The estimation of the purine bases by chemical methods of precipitation or by physical methods (ultraviolet absorption) is open to criticism to a certain extent on account of the variations of these purine bases in nucleic acids of different origins (Chapter 10). Colorimetric methods for the estimation of sugars are liable to interference by ill-defined impurities (Chapter 9). Consequently, when we compare the results obtained by different authors, each of them working with the technique of his choice, we must keep in mind that the absolute values of one author may not be strictly comparable with those of another author; the discrepancies between the results are significant only when the same method has been used.

The first results published by Boivin *et al.*[3] and by Vendrely and Vendrely,[9] which are reported in Table I, were obtained with beef tissues and show two features of interest. First, the DNA content of the nucleus in the different tissues studied seems to be the same; second, the DNA content of the sperm is very approximately half of that in the diploid somatic nuclei in the same species. Mirsky and Ris,[4] working with several animal species, confirmed and extended the view that the sperm cells contain half the DNA content of the somatic cell nuclei (Table II). In the ox, the bull, and the calf, their results were initially not in agreement with those of the French authors, but subsequently Mirsky and Ris revised their early results on cattle[10] and declared themselves in full agreement with the conception of

[6] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.* **161,** 83 (1945).
[7] W. C. Schneider, *J. Biol. Chem.* **161,** 293 (1945).
[8] W. M. McIndoe and J. N. Davidson, *Brit. J. Cancer* **6,** 200 (1952).
[9] R. Vendrely and C. Vendrely, *Experientia* **4,** 434 (1948).
[10] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **34,** 475 (1951).

TABLE I

AMOUNT OF DNA PER NUCLEUS IN BEEF TISSUES[3, 9]

Organ	DNA, pg.[a]	Number of nuclear types	Probable chromosome number
Thymus	6.6	1	Diploid
Liver	6.4	2	Diploid
Pancreas	6.9	6	Diploid
Kidney	5.9	10	Diploid
Sperm	3.3	1	Haploid

[a] 1 picogram (pg.) = 10^{-12} gram.

TABLE II

DNA CONTENT (PICOGRAMS PER NUCLEUS) OF NUCLEI OF DIPLOID CELLS AND SPERM IN SEVERAL SPECIES[4]

Species	Erythrocyte	Liver	Sperm
Domestic fowl	2.34	2.39	1.26
Shad	1.97	2.01	0.91
Carp	3.49	3.33	1.64
Brown trout	5.79		2.67
Toad	7.33		3.70

TABLE III

DNA CONTENTS (PICOGRAMS PER NUCLEUS) OF THE NUCLEI OF FOWL TISSUES[5]

Erythrocyte	2.6	Spleen	2.6
Liver	2.6	Heart	2.6
Kidney	2.4	Pancreas	2.6

constancy of the DNA content of the nucleus in mammals as well as in other groups of animals. Davidson *et al.*,[5] studying the DNA content of the nucleus in several tissues of the fowl, confirmed that the amount of DNA per nucleus was constant in cells from the organs examined (Table III).

The results of the different authors who have studied chemically the DNA content of the nuclei of various tissues from the same animal are shown in Table IV. These results are, in general, in good agreement with the Boivin-Vendrely theory of the constancy of DNA in diploid somatic nondividing nuclei within the same species, this amount being double that found in haploid cells (sperm) in the same species. The striking relationship between the DNA content of the nucleus and the number of chromosomes is clear from this table.

TABLE IV

DNA Content (Picograms per Nucleus) of the Nuclei of Cells of Different Tissues in Various Species

	Cattle		Pig (9)	Dog (14)	Rat				Duck (14)	Fowl		Toad (4)	Carp		Shad (4)	Brown trout (4)	Rainbow trout (18)	Pike (18)	Tench (18)
	(3, 13)[a]	(4)			(5)	(15)	(16)	(17)		(4)	(5)		(14)	(4)					
Liver	6.4	6.22–8.40	5.0	5.3	10.7	10.1	8.2	9.40	2.1	2.39	2.44–2.68		3.0	3.33	2.01				
Thymus	6.4	7.15																	
Kidney	6.4	6–25–6.81	5.2	5.3	7.3	6.7	5.5	6.72			2.31–2.48								
Pancreas	6.6										2.43–2.74								
Spleen	6.8							6.52			2.52–2.66								
Erythrocyte									2.3	2.34	2.55–2.61	7.33	3.3	3.49	1.97	5.79	4.9	1.7	1.7
Leucocyte								6.60											
Lung								6.71											
Intestine								7.60											
Salivary gland								7.55											
Heart								6.46											
Bone marrow								6.90			2.57–2.65								
Sperm	3.3	2.82								1.26		3.70	1.60	1.64	0.91	2.67	2.45	0.85	0.85

[a] Numbers in parentheses indicate references.

But, as some authors have pointed out[11-12] the chemical method of approach must be applied to a large number of nuclei, and the DNA content is calculated from the gross analysis and from the enumeration of the nuclei. The result thus obtained is exact if we assume that all the nuclei of the tissue under consideration have approximately the same DNA content. This conclusion seems to be justified, since nuclei as widely different as those of erythrocytes, liver, pancreas, heart, and so on, have been found to contain the same amount of DNA, an amount double that in the sperm. This striking result cannot be obtained by chance but must reveal a fundamental property of the DNA of the nucleus.

Nevertheless, in certain cases the nuclei of a tissue may not represent a homogeneous population but a mixture of different types. The livers of rodents and some mammals, for instance, are known to contain three kinds of nuclei—diploid, tetraploid, and octoploid—whereas kidney tissue contains only diploid nuclei. In such cases of polyploidy, results for the mean amount of DNA calculated for a single nucleus are markedly higher than in a normal tissue; the ratio between these values for rat liver is 1.4:1 to 1.5:1. These results are mean values and yield, of course, no significant information concerning the real DNA content of the individual nucleus. In such cases, as well as in rapidly growing and differentiating tissues and abnormal or pathological tissues, the techniques of cytophotometry are valuable when chemical methods alone are unable to provide the answer. Cytophotometric methods allow an estimation of the DNA content of individual nuclei, and with their aid the question of the complications due to polyploidy has been clarified.

Before considering the results of photometric methods we must first examine the particular problem of the chemical determination of the DNA content of the egg nucleus. It might be expected that the egg would contain the same amount of DNA as the sperm of the same species. Chemical analyses have usually been carried out on whole eggs, not on isolated nuclei, and the results seem to be obviously wrong; for instance, Schmidt *et al.*,[19] by estimating DNA phosphorus, and Vendrely and Vendrely,[20] measuring

[11] A. W. Pollister, H. Swift, and M. Alfert, *J. Cellular Comp. Physiol.* **38,** Suppl. 1, 101 (1950).

[12] L. Lison and J. Pasteels, *Arch. biol. (Liège)* **62,** 1 (1951).

[13] C. Vendrely, *Bull. biol. France et Belg.* **86,** 1 (1952).

[14] R. Vendrely and C. Vendrely, *Experientia* **5,** 327 (1949).

[15] M. F. Harrison, *Nature* **168,** 248 (1951).

[16] C. Leuchtenberger, R. Vendrely, and C. Vendrely, *Proc. Natl. Acad. Sci. U. S.* **37,** 33 (1951).

[17] R. Y. Thomson, F. C. Heagy, W. C. Hutchison, and J. N. Davidson, *Biochem. J.* **53,** 460 (1953).

[18] R. Vendrely and C. Vendrely, *Compt. rend.* **235,** 444 (1952).

[19] G. Schmidt, L. Hecht, and S. J. Thannhauser, *J. Gen. Physiol.* **31,** 203 (1948).

deoxypentose, found in a single sea urchin egg more than a hundred times as much DNA as in the sperm [*Arbacia* sperm, 21 to 30 pg., egg, 600 to 1000 pg.[19]; *Arbacia acquituberculata* sperm, 0.67 pg., egg, 220 pg.; *Paracentrotus lividus* sperm, 0.70 pg.[20]]. These results are probably due to unavoidable interference by the enormous amount of cytoplasmic substance in the estimation of the very small quantity of DNA of the nucleus. But even more precise methods, such as the microbiological technique used by Hoff-Jørgensen and Zeuthen,[21-22] working on *Rana platyrrhina*, and the microbiological determinations of thymine made by Elson and Chargaff[23] on *Paracentrotus*, yield values for DNA which are still too high (*Paracentrotus lividus* sperm, 1.0 to 1.1 pg., egg, 24 to 26 pg.). As Zeuthen[21] points out, the total amount of DNA found in the frog's egg (0.65 μg., 5000 times more than in the sperm) could not be contained in the nucleus even if it consisted only of a solid mass of DNA. The possibility of extranuclear DNA in eggs must therefore be considered, and the presence of DNA stored in the egg cytoplasm for use in subsequent mitoses is now admitted[21, 22, 24]; cytochemical and biochemical evidence of DNA in the cytoplasm has been provided in several instances.[25-28] This assumption, however, cannot be accepted without reserve, for the egg cytoplasm is generally Feulgen-negative. We must therefore suppose the extranuclear DNA to be in a very dilute state in the cytoplasm and below the limits of stainability; or, alternatively, it may be a precursor of DNA, nonstainable by the Feulgen reaction. This very interesting point needs further investigation and could throw some light on the question of the synthesis of DNA during the first stages of development.

The chemical determination of the DNA content of the nuclei of plant tissues is not easy because the isolation of nuclei is particularly difficult in such material; only the gametes can be studied easily.[29] Heagy and Roper[30] examined the DNA content of conidia of diploid and haploid strains of *Aspergillus nidulans*. They found in diploid strains 9.39 and 7.75 pg. of DNA for 10^9 conidia and in haploid strains 4.04 and 4.22 pg. for 10^9 conidia, i.e., approximately half the content of diploids.

In conclusion, the chemical method for determining the DNA content of

[20] C. Vendrely and R. Vendrely, *Compt. rend. soc. biol.* **143,** 1386 (1949).
[21] E. Hoff-Jørgensen and E. Zeuthen, *Nature* **169,** 245 (1952).
[22] E. Zeuthen, *Pubbl. staz. zool. Napoli* **23,** Suppl. 2, 59 (1952).
[23] D. Elson and E. Chargaff, *Experientia* **8,** 143 (1952).
[24] A. W. Pollister, *Exptl. Cell. Research* **3,** Suppl. 2, 59 (1952).
[25] F. Schrader, *Science* **114,** 486 (1951).
[26] A. H. Sparrow and M. R. Hammond, *Am. J. Botany* **34,** 439 (1947).
[27] N. Fautrez-Firlefyn, *Compt. rend. soc. biol.* **144,** 1127 (1950).
[28] H. Fraenkel-Conrat and E. D. Ducay, *Biochem. J.* **49,** Proc. xxxix (1951).
[29] M. Ogur, R. O. Erickson, G. Rosen, K. B. Sax, and C. Holden, *Exptl. Cell Research* **2,** 73 (1951).
[30] F. C. Heagy and J. A. Roper, *Nature* **170,** 713 (1952).

the nucleus has been applied generally with success to adult tissues where the rate of mitosis is negligible, and to sperm cells.

b. *Results of Histophotometry in Visible Light*

The method of cytophotometry was described as early as 1947 but cytochemical determinations on nuclei could not be,[31] regarded as reliable until they were checked against a chemical procedure. The demonstration of the constancy of DNA in diploid nuclei by chemical methods gave a standard of comparison and permitted a rapid development of the cytophotometric method for the study of a great number of problems. The first cytophotometric studies confirmed the chemical values for the constancy of DNA and its relationship with the number of chromosomes.[32] This work was performed by the school of Pollister (Ris, Leuchtenberger, Swift, Alfert) with the apparatus described by Pollister and Moses.[33] Another apparatus for cytophotometry based upon the same principle was built in Belgium by Pasteels and Lison,[34] whose results have sometimes been in disagreement with those of the American workers, as we shall see later.

Cytophotometric measurements do not permit of a quantitative determination of the DNA content of the nucleus in absolute units, but it is possible to calculate absolute values from the amount in arbitrary units using, as a standard, nuclei of known DNA content treated together with the unknown.[32] In practice, this calculation is of no great interest.

Ris and Mirsky[32] showed the existence in rat liver of three classes of nuclei, the ratio of intensity of the Feulgen reaction being very close to 1:2:4. This occurrence of polyploidy has been confirmed and studied on other organs and other species by Swift,[35] by Pasteels and Lison,[36] by Leuchtenberger and the Vendrelys,[16] and by Davidson.[37]

It would seem generally that an increase in the size of the nucleus is correlated with an increase of the number of chromosomes and consequently with an increase of the DNA content of the nucleus.[32] But Schrader and Leuchtenberger[38] showed that in the insect *Arvelius* the DNA content of the nucleus is the same in three types of spermatocytes, the nuclear volumes of which were 200, 400, and 1600 cubic microns, respectively. Leuchtenberger and Schrader[39] showed also in rat liver this independence of the DNA from the nuclear volume.

[31] A. W. Pollister and H. Ris, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 147 (1947).

[32] H. Ris and A. E. Mirsky, *J. Gen. Physiol.* **33,** 125 (1949).

[33] A. W. Pollister and M. J. Moses, *J. Gen. Physiol.* **32,** 567 (1949).

[34] L. Lison, *Acta Anat.* **10,** 333 (1950).

[35] H. H. Swift, *Physiol. Zoöl.* **23,** 169 (1950).

[36] J. Pasteels and L. Lison, *Compt. rend.* **230,** 780 (1950).

[37] J. N. Davidson, *Bull. soc. chim. biol.* **35,** 49 (1953).

[38] F. Schrader and C. Leuchtenberger, *Exptl. Cell Research* **1,** 421 (1950).

[39] C. Leuchtenberger and F. Schrader, *Biol. Bull.* **101,** 95 (1951).

TABLE V

AVERAGE AMOUNTS OF DNA PER NUCLEUS IN TISSUES OF YOUNG AND ADULT MICE, AS OBTAINED BY PHOTOMETRIC DETERMINATIONS ON FEULGEN PREPARATIONS[35]

Cell types		DNA in arbitrary units	Standard error	Number of nuclei measured
Liver	Class I	3.34	0.05	21
	Class II	6.77	0.07	52
	Class III	13.2	0.25	12
Pancreas	Class I	3.10	0.06	20
	Class II	6.36	0.09	15
	Class III	12.4	—	5
Thymus	Class I	3.28	0.06	33
	Class II	6.17	0.18	21
Lymphocytes	Class I	3.20	0.08	19
	Class II	6.00	0.22	9
Sertoli cells	Class I	3.00	0.12	18
	Class II	6.40	0.26	7
Kidney tubule		3.14	0.04	30
Small intestine epithelium		2.97	0.04	20
Spleen		3.12	0.04	33
Neurones (spinal cord)		3.14	0.07	20
Interstitial cells (testis)		3.05	0.08	20
Spermatids		1.68	0.02	28

Swift[35] made an extensive study of ten different tissues in the mouse. The results, reported in Table V, demonstrate the presence of polyploidy in some organs and the constancy of the DNA content of the diploid nuclei (class I), this common value of DNA being twice that found in spermatids. This is in good agreement with the figures from chemical analysis reported above. This relationship of DNA to the number of chromosomes is evident also during the formation of gametes. During spermatogenesis, primary spermatocytes contain four times the amount of DNA found in the spermatids, as is consistent with the fact that one primary spermatocyte will give four spermatids after the two meiotic divisions. This fact has been demonstrated by Lison and Pasteels in *Talpa*,[40] by Schrader and Leuchtenberger in *Arvelius albopunctatus*[38] and by Swift in the mouse.[35] An identical process was described by Alfert[41] in oögenesis in the mouse; he found that pronuclei resulting from two meiotic divisions carry one-fourth of the DNA that is contained in primary oöcytes.

The results originally obtained by Pasteels and Lison[36] on different organs of the rat seem to be in disagreement with those of the American workers; the DNA content of the nuclei of some organs (liver, pancreas, adipose

[40] L. Lison and J. Pasteels, *Compt. rend. soc. biol.* **143**, 1607 (1949).
[41] M. Alfert, *J. Cellular Comp. Physiol.* **36**, 381 (1950).

tissue) was claimed to be markedly lower than the expected value (twice that of the spermatid). A similar examination of rat tissues was made by Leuchtenberger *et al.*[16] and also by Ris,[32] and by Swift[35] with Pollister's apparatus, all of whom found none of the discrepancies reported by Pasteels and Lison. The apparent discrepancy has now been explained in a recent publication by Pasteels and Lison,[42] who, in a new series of measurements made on other rats (their first experiment was on only one animal), have obtained results in agreement with the cytochemical and chemical observations of other workers. The single rat studied previously by Pasteels and Lison appears to have been exceptional. A complete study of such a case would be of the greatest interest, but unfortunately no other rat of this type has been found at the present time.

c. *Quantitative Photometry in Ultraviolet Light*

The absorption of ultraviolet light as in Caspersson's apparatus (Chapter 17) has been used recently for a quantitative estimation of the DNA of nuclei.[43] The absorption of light at 260 mμ in the nucleus is due to the DNA of the chromatin and also to the PNA of the nucleolus and to their nucleotides, but the error caused by these last two groups in the measurement of DNA does not seem to be very high. If the nuclei are fixed, most of the fixatives dissolve simple acid-soluble nucleotides; and if the nuclei are isolated, the isolation procedure with citric acid removes a great part of the PNA. Nevertheless, Frazer and Davidson[44] showed that crystalline ribonuclease removes a considerable proportion of the total material absorbing ultraviolet light, so that absorption measurements are much more precise after the use of the enzyme. In any case, photometry in visible or in ultraviolet light cannot reach a high degree of precision, and a certain amount of error is unavoidable.

Up to the present time, few results on nuclei have been obtained with photometry in ultraviolet light. Leuchtenberger *et al.*[43] studied isolated nuclei from beef liver, calf thymus, and bull sperm; the results calculated in absolute amounts of DNA are in reasonable agreement with the values from chemical estimation. In rat liver, the three classes of nuclei were found as with photometry in visible light. Walker and Yates[45, 46] reported results obtained on living cells in tissue culture and on living erythrocytes and sperm. Their figures are in agreement with chemical results. Frazer and Davidson,[44] working on nuclei isolated from rat kidney and rat liver, chose

[42] J. Pasteels and L. Lison, *Compt. rend.* **236,** 236 (1953).
[43] C. Leuchtenberger, R. Leuchtenberger, C. Vendrely, and R. Vendrely, *Exptl. Cell Research* **3,** 240 (1952).
[44] S. C. Frazer and J. N. Davidson, *Exptl. Cell Research* **4,** 316 (1953).
[45] P. M. B. Walker and H. B. Yates, *Proc. Roy. Soc.* (*London*) **B140,** 274 (1952).
[46] P. M. B. Walker and H. B. Yates, *Symposia Soc. Exptl. Biol.* **6,** 265 (1952).

to express their measurements in arbitrary units. Their results confirmed the homogeneity of the nuclear population of the kidney and the presence of at least two classes of nuclei in liver.

These early investigations show that cytophotometry in ultraviolet light can be used successfully for the study of the DNA content of nuclei.

2. The DNA Content of the Nucleus in Different Species of Animals

Since the DNA content of the nucleus appears as a constant value characteristic of the species, a comparison of the amounts of DNA per nucleus throughout the animal kingdom is of some interest. Numerous results are already available (Tables VI and VII), but their interpretation is not easy. Nevertheless, a few points can be stressed. Mammals and birds so far studied show values of DNA per nucleus which are confined within narrow limits. Mirsky and Ris[47] remark that the values of DNA per cell in lung fish, amphibians, and reptiles suggest that in the evolution of these vertebrates there has been a decline in the DNA content per cell. On the other hand, in invertebrates, the amount of DNA per cell is greater in higher forms than in primitive forms. Vendrely and Vendrely[48] suggest that some high values found in fishes could represent polyploids, but sufficient information is not yet available to settle the question.

In conclusion, can the study of the DNA content of the nucleus be of some help in problems concerning evolution? The quantity of DNA does not seem to be related to the number of genes, for the amount of DNA does not increase unequivocally with the complexity and number of hereditary characters. It should, however, be pointed out that less organized living beings seem to have small amounts of DNA in their nuclei: Boivin *et al.*[3] estimated that the DNA content of a bacterial nucleus represented about one-hundredth that of the beef nucleus, and Whitfeld[49] recently found a value of the same order of magnitude (0.059 pg.) in *Plasmodium berghei*. But, within the same species, the amount of DNA per nucleus is certainly related to the number of chromosomes, and it may well be that in related species the differences in the DNA content of nuclei could be correlated with differences in the size and the total length of the chromosomes. The precise enumeration of the chromosomes is sometimes very complex on account of fragmentation and other difficulties. In such cases it is readily possible to obtain additional information from the measurement of DNA. Therefore the DNA complement can be considered, as Hughes-Schrader[50] points out, as "a cytotaxonomic tool in evaluating evolutionary relationship among species

[47] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **34**, 451 (1951).
[48] R. Vendrely and C. Vendrely, *Compt. rend.* **230**, 670 (1950).
[49] P. R. Whitfeld, *Nature* **169**, 751 (1952).
[50] S. Hughes-Schrader, *Biol. Bull.* **100**, 178 (1951).

TABLE VI

DNA Content (Picograms per Nucleus) of Somatic Cells of Various Vertebrates

	Species	DNA (47)[a]	(14, 13)	(52)	(8)
Dipnoan	African lungfish (*Protopterus*)	100			
Amphibians	Amphiuma	168			
	Necturus	48.4			
	Frog	15.0			
	Toad	7.33			
Reptiles	Green turtle	5.27			
	Wood turtle	4.92			
	Snapping turtle	4.97			
	Alligator	4.98			
	Water snake	5.02			
	Pilot snake	4.28			
	Black racer snake	2.85			
Birds	Domestic fowl	2.34	2.2		2.3
	Guinea hen	2.27			
	Goose	2.92	1.9		
	Duck	2.65	2.2		
	Pigeon		2.0		
	Turkey		1.9		
	Pheasant		1.7		
	Sparrow		1.9		
Mammals	Ox		6.4	6.9	7.1
	Pig		5.1	6.8	
	Guinea pig		5.9		
	Dog		5.3	6.7	
	Man		6.0	6.8	
	Rabbit		5.3		6.5
	Horse		5.8		
	Sheep		5.7	6.8	
	Rat		5.7		
	Mouse		5.0		

[a] Numbers in parentheses indicate references.

whose karyotypes are not analysable by the method of comparative cytology." This author has studied the relative amounts of DNA per spermatid nucleus in eight species of *Mantidae* by photometric measurements and compared this amount with the number of chromosomes and their size (total length in arbitrary units). In one case this comparison suggested the possibility of polyploidy in the evolutionary process; in other cases, the DNA content was the same in two species in which the number of chromosomes was very different and in which polyploidy was evident. Moses and Yer-

TABLE VII

DNA CONTENT (PICOGRAMS PER NUCLEUS) OF FISH ERYTHROCYTES

	Species	DNA (47)[a]	(13, 14)
Agnatha	Lamprey (*Petromyzon*)	5	
Elasmobranchii	Shark (*Carcharias obscurus*)	5.46	
	Shark (*Carcharias longimanus*)	6.67	
Chondrostei	Sturgeon (*Acipenser sturio*)	3.2	
Holostei	Bowfin (*amia*)	2.3	
Teleostei			
Clupeidae	Pilchard (*Harengala sardinia*)	2.04	
	Shad (*Alosa*)	1.97	
Cyprinidae	Carp (*Cyprinus carpio*)	3.49	3.2
	Barbel (*Barbus barbus*)		3.4
	Roach (*Gardonus rutilus*)		1.9
	Tench (*Tinca tinca*)		1.7
Percidae	Perch (*Perca fluviatilis*)		1.9
Esocidae	Pike (*Esox lucius*)		1.7
Salmonidae	Rainbow trout (*Salmo irideus*)		4.9
Anguilidae	Eel (*Anguilla anguilla*)		1.9
Siluridae	Catfish (*Ameiurus nebulosus*)	1.89	1.8
Scaridae	Rainbow parrot (*Pseudoscarus guacamaia*)	2.5	
	Red tailed parrot (*Sparisoma brachiale*)	2.45	
	Mudbelly (*Scarus croicensis*)	2.58	
Balistidae	Tiggerfish (*Babistes capriscus*)	1.07	
Carangidae	Bonito (*Zonichthys faleatus*)	1.35	
	Jack (*Caranx lactus*)	1.21	
	Round robin (*Decapterus punctatus*)	1.32	
Acanthuridae	Doctor fish (*Acanthurus hepatus*)	1.38	
Ostraciontidae	Cow fish (*Lactophrys quadricornis*)	1.91	
Aulostomatidae	Trumpet fish (*Aulostomus maculatus*)	1.39	
Holocentridae	Squirrel fish (*Holocentrus ascensionis*)	1.31	
Mugilidae-Sphyraenidae	Mullet (*Mugil curcma*)	1.38	
	Barracuda (*Sphyraena barracuda*)	1.37	
Sparidae	Sheepshead porgy (*Calamus calamus*)	2.24	
	Bream (*Diplodus argenteus*)	1.61	
Haemulidae	Blue striped grunt (*Haemulon sciurus*)	1.20	
	Yellow grunt (*Haemulon flavilineatum*)	1.33	
Gerridae	Shad (*Eucinostomus guba*)	0.94	
Lutianidae	Yellow tail (*Ocyurus chrysurus*)	2.1	
	Gray snapper (*Lutianus griseus*)	2.1	
	Silk snapper (*Lutianus hastingsi*)	2.1	
Belonidae	Hound fish (*Tylosurus acus*)	2.2	
Serranidae	Red hind (*Epinephelus guttatus*)	2.09	
	Butterfish (*Cephalopholis fulvus*)	1.93	
	Hamlet (*Epinephelus striatus*)	2.05	
	Gay (*Mycteraperca tigris*)	2.2	
	Monkey rockfish (*Trisotropis venenosus*)	1.83	

[a] Numbers in parentheses indicate references.

ganian,[51] studying the DNA content of nuclei in various tissues of two species of hamsters with a very different chromosome number, suggested that chromosome fragmentation rather than duplication and polyploidy was the mechanism which must be evoked to explain the chromosomal evolution of these *Cricetidae*.

[51] M. J. Moses and G. Yerganian, *Records Genet. Soc. Amer.* **21,** 51 (1952).

[52] P. Metais, S. Cuny, and P. Mandel, *Compt. rend. soc. biol.* **145,** 1235 (1951).

III. Can the DNA Content of the Nucleus be Considered as a "Constant"?

1. Interpretation of the Notion of Constancy in Biology

The results quoted above suggest strongly that the DNA content of the nucleus is a constant characteristic of the species and, within the species, related to the number of chromosomes. From the theoretical point of view is it possible to speak of "constancy" of the amount of DNA in the nucleus in one animal as one speaks of constancy for the number of chromosomes? This does not, of course, mean that the DNA content of the nucleus is always the same during the whole life of the cell. Each substance in the cell is involved in a cycle of anabolic and catabolic processes, and, especially before mitosis, a certain quantity of DNA must be synthesized so that the two daughter cells contain the same amount of DNA as did the mother cell. Even during interphase, one can imagine that the DNA may undergo some variation in relation to the physiology of the cell. In resting nuclei, however, it seems that these variations in general are slight and do not appreciably affect the total amount of DNA in the nucleus. Studies made with isotopes[53-56] have shown that the turnover of DNA is very slow compared to the turnover of PNA (Chapters 25 and 26), so that variations most likely occur only in a small number of molecules at a time without any change in the quantity of DNA in interphase nuclei.

On the other hand, the physiological changes which sometimes act very strongly upon PNA do not affect the DNA content of the nuclei. Fasting or protein-deficient diets which produce a considerable decrease of PNA in rat liver have no significant influence on the DNA content of the liver nucleus, which remains unchanged even in extreme cases of prolonged fasting and prolonged consumption of protein-free diets, as was shown by Mirsky and Kurnick,[57] McIndoe and Davidson,[8] Campbell and Kosterlits,[58] Villela,[56] Thomson *et al.*,[17] and Fukuda and Sibatani.[59] In young animals which are still growing, it seems that a protein-deficient diet produces a slight increase of the DNA content of the nuclei.[58, 60, 61] This change could be explained, as Thomson *et al.* point out,[17] by the fact that protein deficiency in a still-growing animal might inhibit growth and mitosis to a greater extent than it affects the premitotic synthesis of DNA, and this

[53] A. M. Brues, M. M. Tracy, and W. E. Cohn, *J. Biol. Chem.* **155,** 619 (1944).
[54] E. Hammarsten and G. Hevesy, *Acta Physiol. Scand.* **11,** 335 (1946).
[55] G. B. Brown, M. L. Petermann, and S. S. Furst, *J. Biol. Chem.* **174,** 1043 (1948).
[56] G. G. Villela, *Rev. brasil. biol.* **12,** 321 (1952).
[57] A. E. Mirsky and N. B. Kurnick, quoted by A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **34,** 451 (1951).
[58] R. M. Campbell and H. W. Kosterlitz, *Science* **115,** 84 (1952).
[59] M. Fukuda and A. Sibatani, *Exptl. Cell Research* **4,** 236 (1953).
[60] J. O. Ely and M. H. Ross, *Science* **114,** 70 (1951).
[61] C. Lecomte and A. de Smul, *Compt. rend.* **234,** 1400 (1952).

could result in an apparent increase in the average DNA content per nucleus.

The action of hormones does not seem to affect seriously the DNA content of nuclei. Alfert and Bern[62] showed that the injection of estrogen into ovariectomized rats does not change the DNA of the nuclei in the uterine gland cells although the protein content is doubled. Di Stephano *et al.*[63] noted that hypophysectomy does not change the DNA content of the nuclei of rat liver. But Bergerard and Tuchmann-Duplessis[64] found a slight decrease in the DNA content of nuclei B (tetraploid) in rat liver after hypophysectomy, the injection of somatotrope hormone bringing these nuclei back to a normal value. The fact that these authors worked on young rats might perhaps explain their discrepancy with Di Stephano *et al.*[63]

Fukada and Sibatani[65] noted that the DNA content of nuclei in the guinea pig liver is not affected by experimental ascorbic acid deficiency. A thiamine-deficient diet, a high-fat diet, administration of a diabetogenic dose of alloxan, or administration of thioacetamide does not change the DNA content per nucleus in rat liver (Thomson *et al.*[17]).

Factors such as age, sex, strain, and body weight[17] have no noticeable influence upon the amount of DNA per nucleus, but we must note here that the degree of polyploidy is higher in adult rats than in young animals.[17, 35]

Finally, it should be noted that the onset of pathological changes does not seem to affect directly the DNA of the nucleus, which remains unchanged until an advanced stage of cell degeneration. Leuchtenberger,[66] studying pycnotic degeneration of neoplastic and normal nuclei in mice, showed that the DNA content is unchanged in an advanced state of pycnosis whereas the nucleus has already lost an important part of its protein content, nor is the amount of DNA per nucleus affected even in cases where the amounts of PNA, protein, and other substances are considerably altered.

Mark and Ris[67] showed that the nuclei of cholangioma and hepatoma have the same content of DNA as have normal rat liver nuclei. Cunningham *et al.*, [68, 69] studying normal, precancerous, and neoplastic rat tissues found in all these cases the same average value of DNA per nucleus; the precancerous changes were obtained by feeding with acetylaminofluorene or

[62] M. Alfert and H. A. Bern, *Proc. Natl. Acad. Sci. U. S.* **37**, 202 (1951).
[63] H. S. Di Stephano, A. D. Bass, H. F. Diermeier, and J. Tepperman, *Endocrinology* **51**, 386 (1952).
[64] J. Bergerard and H. Tuchmann-Duplessis, *Compt. rend.* **236**, 1080 (1953).
[65] M. Fukuda and A. Sibatani, *Experientia* **9**, 27 (1953).
[66] C. Leuchtenberger, *Chromosoma* **3**, 449 (1950).
[67] D. D. Mark and H. Ris, *Proc. Soc. Exptl. Biol. Med.* **71**, 727 (1949).
[68] L. Cunningham, A. C. Griffin, and J. M. Luck, *Cancer Research* **10**, 211 (1950).
[69] L. Cunningham, A. C. Griffin, and J. M. Luck, *J. Gen. Physiol.* **34**, 59 (1950).

3′-methyl-4-dimethylaminoazobenzene. Price and Laird[70] and Price *et al.*[71] could not demonstrate any difference between the nuclei of induced liver tumors and normal liver in the rat. Davidson *et al.*[72,73] found no difference between the DNA content of nuclei of normal and of leukemic bone marrow cells, but they noticed a significant rise in DNA in pernicious anemia. Metais and Mandel[74] found the same DNA content in human leukemic cell nuclei and normal leucocyte nuclei.

On the other hand, McIndoe and Davidson[8] have found in a fowl sarcoma an amount of DNA per nucleus higher than the amount in the normal fowl nucleus. Klein and Klein[75, 76] studied different tumors in the mouse and found that some contained the normal amount of DNA per cell whereas others showed higher values. Further investigations by Leuchtenberger *et al.*[77] by ultraviolet microspectrophotometry showed that the DNA content per cell in Ehrlich ascites tumor cells is approximately that of tetraploid nuclei; the relative deviations from the mean value do not differ significantly from those found in normal cells, while the DBA ascites lymphoma nucleus contains the normal diploid amount of DNA.

To summarize, the DNA content of the nucleus does not seem to be affected by carcinogenic drugs and the neoplastic process; only a few cases of variations in DNA content have been found in neoplasms and some can be related to polyploidy. We can therefore say that in normal tissues as well as in tissues undergoing drastic physiological and pathological changes, the DNA of resting nuclei is remarkably stable. But it is possible that some individual changes may occur among the population of cells thus studied. The results of photometric analysis on a great number of nuclei cover a certain range of values, and it is sometimes very difficult to decide whether these differences are due to errors inherent in the technique or whether they are biologically significant and illustrate real variations in the nucleus. It might well be, for instance, that a degenerative process is at work when the value found is markedly lower than the theoretical value; or a synthesis of DNA in preparation for future mitosis might account for a high value. This possibility needs further investigation.

A few examples are known, however, where the DNA per nucleus is definitely different from the normal value.

[70] J. M. Price and A. K. Laird, *Cancer Research* **10,** 650 (1950).

[71] J. M. Price, E. C. Miller, J. A. Miller, and G. M. Weber. *Cancer Research* **10,** 18 (1950).

[72] J. N. Davidson, I. Leslie, and J. C. White, *Lancet* (i), 1287 (1951).

[73] J. N. Davidson, I. Leslie, and J. C. White, *J. Pathol. Bacteriol.* **63,** 471 (1951).

[74] P. Metais and P. Mandel, *Compt. rend. soc. biol.* **144,** 277 (1950).

[75] Eva Klein and G. Klein, *Nature* **166,** 832 (1950).

[76] G. Klein, *Exptl. Cell Research* **2,** 518 (1951).

[77] C. Leuchtenberger, G. Klein, and E. Klein, *Cancer Research* **12,** 480, (1952).

2. Possible Variations of the DNA Content of the Nucleus

a. *DNA and Polyteny*

Schrader and Leuchtenberger[78] have reported in the plant *Tradescantia* different values for the DNA content, determined by photometry, of the interphase nuclei of the root tip (5.5 in arbitrary units), leaf (9.0), and bud tapetum (12.0), although the number of chromosomes is the same for all these tissues. This surprising result can be explained, as these authors point out, by the occurrence of polyteny in such tissues. The chromosomes are composed of a number of threads (chromonemata); if each thread carries a definite amount of DNA, the reduplication of these chromonemata (polyteny) must lead to an increase of the DNA content of the nucleus without any increase in the number of chromosomes. The results of Swift[79] suggest that a process of synchronous reduplication in all the chromosomes is concerned (endomitosis), for the values found are exact multiples of the diploid amount in nondividing tissues. But, from the figures of Schrader and Leuchtenberger[78] and of Bryan[80] on *Tradescantia*, and of Huskins and Steinitz[81] on *Rhoeo*, it appears that the process involved in plants must be a partial reduplication of the chromosomal set.

The process of reduplication of chromonemata occurs very strikingly in the well-known giant chromosomes of the salivary gland of *Drosophila*. Kurnick and Herskowitz[82] have found in *Drosophila* salivary gland nuclei DNA values which vary progressively from 0.56 to 71.2 pg. The limb anlage cell nucleus selected as a suitable diploid nucleus for the determination of the base value in *Drosophila* contains about 0.17 pg. of DNA. These results have been obtained by photometry using methyl green for the determination of DNA. This 420-fold increase in DNA content in the largest salivary gland nuclei would represent 840 chromomonemata per double salivary chromosome.[83]

The findings of Moses[84] on *Paramecium caudatum* give precise information on the chemical nature of the two nuclei of these Protozoa: the macronucleus and the micronucleus contain similar concentrations of DNA, PNA, total protein, and nonhistone protein, but the macronucleus carries a multiple (of the order of 40×) of the DNA of the micronucleus, which would contain the diploid amount of genetic elements.

[78] F. Schrader and C. Leuchtenberger, *Proc. Natl. Acad. Sci. U. S.* **35,** 464 (1949).
[79] H. H. Swift, *Proc. Natl. Acad. Sci. U. S.* **36,** 643 (1950).
[80] J. H. D. Bryan, *Chromosoma* **4,** 369 (1951).
[81] G. L. Huskins and L. M. Steinitz, *J. Heredity* **39,** 34 (1948).
[82] N. B. Kurnick and I. H. Herskowitz, *J. Cellular Comp. Physiol.* **39,** 281 (1952).
[83] E. K. Petterson and M. E. Dackerman have adapted a micromethod for the chemical study of the DNA of *Drosophila* salivary glands; the amount which they found per cell was 284 pg. DNA. [*Arch. Biochem. and Biophys.* **36,** 97 (1952)].
[84] M. J. Moses, *J. Morphol.* **87,** 493 (1950).

b. *Pollen Formation*

The problem of the formation of the pollen grain is not quite clear at the present time. It has been studied photometrically by Schrader and Leuchtenberger,[78] Bryan,[80] and Pasteels and Lison[85] on *Tradescantia* and by chemical methods by Ogur *et al.*[29] on *Lilium longiflorum*. All these authors have pointed out that the late microspore contains an amount of DNA much higher than the expected value for a haploid cell. Yet, just before prophase, in the microsporocyte the DNA content of the nucleus represents four times the haploid value. This amount of DNA is exactly shared in the four following microspores, but there is a postmeiotic increase in this DNA,[80, 85] so that the microspore nucleus finally contains an amount of DNA of the same order of magnitude as does the diploid nucleus of the root tip or of the bud scale epidermis.[78, 80, 85] The microspore mitosis occurs at this stage, and in the two nuclei thus formed in the pollen grain—the vegetative nucleus and the germinative nucleus—there is a further gradual increase in the DNA content. According to Pasteels and Lison[85] this postmitotic increase of the DNA occurs only in the generative cell nucleus which contains the diploid value. Bryan[80] could not measure the DNA in the differentiating generative nucleus, but he described an increase of the DNA in the vegative nucleus. Ogur *et al.*[29] by gross chemical analysis, have shown that at anthesis the pollen grain contains a very high value of DNA (about eight times the haploid value).

It is difficult to draw any conclusion from these few investigations on the formation of the pollen grain, but it is possible that the biochemical process involved here is somewhat different from that of spermatogenesis in animals. On the whole, the general relation between the number of chromosomes and the DNA content of the nucleus is not always so exact in plant as in animal nuclei. The interpretation of this phenomenon (polyteny, or other processes) is still under consideration.

c. *DNA and Metabolism*

The possibility of extrusion of DNA (Feulgen-positive material) out of the nucleus into the cytoplasm has been described in some cases. Lison and Fautrez-Firlefyn[27, 86] have reported in certain oöcytes of a crustacean, *Artemia salina*, an appreciable increase in the DNA of the nucleus which they think represents a considerable degree of polyploidy. It is followed by an extrusion of the DNA out of the nucleus and the death of the cell. According to these authors, the exaggeration of the process of polyploidy (to eight times and more the haploid value) would be a lethal phenomenon for cell. On the other hand, Schrader and Leuchtenberger,[87] working on the end

[85] J. Pasteels and L. Lison, *Compt. rend.* **233,** 196 (1951).
[86] L. Lison and N. Fautrez-Firlefyn, *Nature* **166,** 610 (1950).
[87] F. Schrader and C. Leuchtenberger, *Exptl. Cell Research* **3,** 136 (1952).

chambers of the ovarial lobes of one of the Hemiptera, described extrusions of DNA out of the nucleus in some cells as participation of this DNA in the formation of nutritive substances which are transferred to the eggs. The very detailed description of these authors shows that the considerable increase of the DNA per nucleus (up to seventeen times) results probably from the fusion of several nuclei (instead of the process of endomitosis suggested by Lison and Fautrez-Firlefyn). Finally, Leuchtenberger and Schrader[88] have found in the salivary gland of *Helix aspersa* a striking example of considerable variation of the DNA content per nucleus (from 30 to 1) correlated with the production of secretory products in the cytoplasm.

The considerable variations of the DNA content of the nucleus reported here seem to be rather rare. They have been described only in insects and lower organisms; nothing similar has been found in vertebrates. When these processes do not represent some degree of polyploidy, they can be interpreted as a particular case of direct participation of the DNA of the nucleus in the secretory process of the cell. The enormous synthesis of DNA in such nuclei, which are not going to divide, is of great interest and could throw some light on the physiological role of the nucleus.

3. The DNA Content of the Nucleus in Dividing Cells

a. *Preparation for Mitosis and Meiosis*

We must now consider the problem of the DNA content of dividing nuclei. Since the two daughter cells have the same amount of DNA in their nuclei as the mother cell, cell division must be preceded or accompanied by a synthesis of DNA. When does this synthesis occur? The exact moment is still a matter of argument. The earliest observations come from Caspersson's ultraviolet work[89] on the spermatogenesis of the grasshopper. They suggested that the synthesis of DNA occurs during the first phases of division and reaches its maximum at metaphase. Ris,[90] on the other hand, studied mitosis in terminal meristems of onion root tips and spermatogenesis of *Chorthophaga* by the photometric technique in visible light and obtained results in agreement with Caspersson's: the synthesis of DNA would occur at prophase and would be complete at metaphase when the total amount of DNA of the chromosomes was twice that of a normal nucleus. But more recent work indicates that the synthesis of DNA must occur earlier. When the nucleus begins its morphological changes characterizing prophase, it should already show the DNA content of the two future nuclei.

It should be pointed out that a number of authors[35, 38, 40] have shown that, in meiosis, the first spermatocyte already contains twice the amount of DNA of the diploid nuclei, i.e., all the DNA necessary for the four sperma-

[88] C. Leuchtenberger and F. Schrader, *Proc. Natl. Acad. Sci. U. S.* **38,** 99 (1952).
[89] T. Caspersson, *Chromosoma* **1,** 147 (1939).
[90] H. Ris, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 158 (1947).

tids which will be derived from it; on the other hand, Alfert[41] has found in primary oöcytes before meiosis four times as much DNA as in the pronuclei resulting from the two meiotic divisions.

In an investigation on mitosis, Swift[35] studied the behavior of DNA in the nuclei of developing liver tissue of the 11-day mouse embryo and in the pronephros and erythrocyte nuclei of the recently hatched *Amblystoma* larva. He noted for interphase nuclei DNA values spreading between the normal diploid value and twice this amount. The posttelophase nuclei presented diploid values, whereas the early prophase nuclei had twice the diploid value. The synthesis of DNA must therefore occur during interphase before the visible stages of mitosis. Seshachar[91] found a similar process in the division of the ciliate micronucleus. Alfert,[41] studying the early development of mouse embryo, found that the DNA content of the nuclei is always doubled prior to the onset of nuclear division.

Finally, Walker and Yates[45] have studied by ultraviolet absorption and by the Feulgen method the DNA content of nuclei in tissue cultures . These authors were able to determine the exact phase of the cell under consideration by studying its history in a phase contrast film taken previously during all the developmental stages. They found that posttelophase nuclei contain the same amount of DNA as erythrocyte nuclei, i.e., the diploid amount. This amount is doubled during interphase and reaches its maximum before prophase. This study upon living cells, the precise stage of development of which can be accurately determined, seems to be more reliable than results from fixed and stained material. It therefore seems well established that the synthesis of DNA occurs during late interphase, before prophase. Nevertheless, Pasteels and Lison,[92] working upon erythroblasts of the rat embryo, Lieberkühn glands of the adult rat, and fibroblasts of the chick heart embryo in tissue culture have found that in these rapidly growing cells the DNA content of the nucleus does not change during prophase. At anaphase the two daughter nuclei contain half this value, but the initial value is reached again at telophase, so that the synthesis of the DNA would appear to occur in telophase and would be completed during the reconstruction of the daughter nuclei. These results are in complete disagreement with the conclusions of other authors. In Fig. 1 are summarized the different theories for the synthesis of DNA. Further work is necessary to clear up the discrepancies, but it should be pointed out, as Walker and Yates[45] have done, that Pasteels and Lison compared spherical interphase nuclei and the irregular filamentous area of the chromosomes. Errors in photometric measurements are unavoidable in such cases, and Pasteels and Lison themselves stress the great difficulty of such work. The concep-

[91] B. R. Seshachar, *Nature* **165**, 848 (1950).

[92] J. Pasteels and L. Lison, *Arch. biol.* (*Liége*) **61**, 445 (1950).

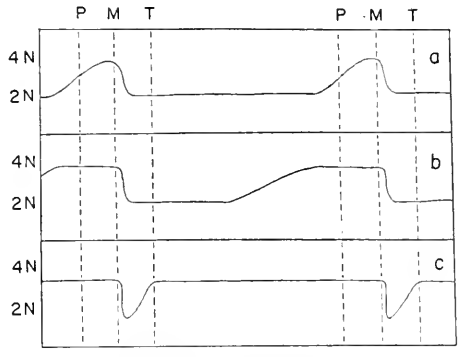

FIG. 1. Development of the DNA content of the cell in the course of mitosis: (*a*) theory of Caspersson[89] and Ris[90]; (*b*) the generally accepted theory (Swift[35], Alfert[41], Walker and Yates[45]; (*c*) theory of Pasteels and Lison.[92]

tion of the synthesis of DNA in late interphase seems to be accepted by most authors at the present time.

b. *DNA of the Nuclei in Embryonic Tissues and Tissue Cultures, and the Problem of Differentiation*

Whatever be the moment when the synthesis of DNA occurs, cell division is associated with a doubling of the DNA content, so that the DNA of the nuclei in actively growing tissues (embryos, tissue cultures, regenerating tissues, etc.) must be higher than in resting tissues. This is evident from the gross chemical analysis of Thomson *et al.*[17] on eleven different organs of the rat. Salivary gland and intestine, where the rate of mitosis is higher than in other tissues, show a higher average amount of DNA per nucleus. In liver regenerating after partial hepatectomy the average amount of DNA per cell is increased markedly during the first days when the rate of growth and multiplication is extremely high.[17, 70] Lison and Pasteels[12] studied cytophotometrically the DNA content of the nuclei in developing sea urchin embryos and noted particularly high values in the actively dividing parts of the embryo. Swift[35] also reported in dividing cells amounts of DNA higher than the values found in resting nuclei and attributed these variations to the building up of DNA for future mitosis. This interpretation seems to be quite logical, but Lison and Pasteels[12] advanced the view that the increase of DNA in dividing cells is not exactly related to variations in the mitotic rhythm. They consider that some changes in the DNA per cell could be caused by the cytoplasmic factors acting on morphogenesis. The results of Moore[93] on embryos of *Rana pipiens* seem also to indicate a correlation between differentiation and a wide range of DNA values in a tissue.

[93] B. C. Moore, *Chromosoma* **4**, 563 (1952).

His mitotic counts showed that there is no visible correlation between DNA content of nuclei and mitotic activity. Moreover, the well-differentiated tissues of the forebrain of the 11-day embryo show a much narrower range of DNA values per nucleus than do those of 7-day embryos which are undergoing differentiation. In the adult cells of *Rana pipiens* studied by Swift,[35] when differentiation is quite finished, the results of DNA determination per nucleus are very close to each other and show a real constancy. Marinone,[94] studying the differentiation of erythroblasts and granuloblasts in man, observed a marked decrease in nuclear DNA in the course of differentiation. This decrease was not related to pycnosis for it appeared abruptly at the stage of the disappearance of the nucleoli. A further and more gradual decrease occurred subsequently, and the nucleus finally contained the diploid amount of DNA. At the end of this process any further decrease in DNA was due to pycnosis and the final amount of DNA might be lower than in lymphocytes. Reisner and Korson[95] also showed a decrease of the DNA content of erythroblasts in the course of differentiation. All these results suggest that differentiation is associated with a variation in the DNA content of the nucleus. In conclusion, the hypothesis of Moore,[93] who interprets the variations of DNA in differentiating cells as a sign of the morphogenetic activity of genes, should be mentioned. This would explain how cells with the same chromosome complement differentiate into quite different tissues. The genes controlling certain morphogenetic processes may produce different amounts and kinds of DNA at different times with the result that differentiation takes place.

IV. Conclusion

With the exception of the few particular cases that have just been reported, the DNA content of the resting nucleus appears as a constant value related to the number of chromosomes, and this striking fact is of great interest for the biologist from the theoretical as well as the practical point of view. From the theoretical point of view the discovery of the constancy of the amount of DNA per nucleus in all tissues of the same animal and the fact that the sperm contains half the DNA content of somatic cells is confirmation of the theory that DNA is an important component of the gene. From the practical point of view Davidson and Leslie[96, 97] have stressed the importance of the constant amount of the DNA per nucleus as a measure of cell multiplication and as a standard of reference in the expression of biochemical changes in tissues. The results of tissue analysis are thus re-

[94] G. Marinone, *Le Sang.* **22,** 89 (1951).
[95] E. H. Reisner and R. Korson, *Blood* **6,** 344 (1951).
[96] J. N. Davidson and I. Leslie, *Nature* **165,** 49 (1950).
[97] J. N. Davidson and I. Leslie, *Cancer Research* **10,** 587 (1951).

ferred to the single average cell and give a more realistic and significant expression of the phenomena. Davidson and his colleagues have carried out an exhaustive study of the variations of PNA, phospholipids, and proteins in embryos at different stages of development and in animals submitted to nutritional changes (Chapter 16).

We have also seen that the DNA value per nucleus can be used in problems related to the evolution of closely related species and in the examination of the possibility of polyploidy in the evolutionary process. Finally, DNA can be used in a comparative chemical study of the other components of the nucleoprotein of the nucleus and their possible variations.

The DNA content of the nucleus therefore appears at present to be a very convenient tool for biologists in different fields, since it allows the study of biochemical processes at the level of the individual cell.

V. Addendum

The fact that the DNA content of the cell nucleus is constant per set of chromosomes in the rat was confirmed recently by Thomson and Frazer,[98] who found that the coefficient of variation in the DNA content of individual nuclei of the same class probably does not exceed 5 to 15%. Alfert and Swift[99] attributed the discrepancies reported by Pasteels and Lison[42] in rat liver to a different degree of fixation in the blocks of tissue from the edge to the center. In rat liver nuclei isolated from sucrose homogenates, the diminution reported by Pasteels and Lison is no longer found. Patau[100] also reached the conclusion that there is no variation in DNA between nuclei with equal chromosome complements.

That the DNA content per nucleus is not affected by physiological changes has been confirmed by Laird,[101] who reported that thioacetamide—which provokes considerable changes in the rat liver nucleus (an increase in protein and PNA)—has no effect on the DNA content. Phillips *et al.*[102] showed that the treatment of sexually immature pullets with gonadal hormones increases the number of liver cells but does not affect significantly the DNA per nucleus. However, Fautrez and Moerman,[103] studying the DNA content of the liver nuclei in the fish *Lebistes reticulatus*, concluded that the variations from the normal diploid value were related to the changes in the physiological activity of the organ, and Govaert,[104] using the vitellogen

[98] R. Y. Thomson and S. C. Frazer, *Exptl. Cell Research* **6**, 367 (1954).
[99] M. Alfert and H. Swift, *Exptl. Cell Research* **5**, 455 (1953).
[100] K. Patau, *Records Genet. Soc. Amer.* **22**, 90 (1953).
[101] A. K. Laird, *Arch. Biochem. and Biophys.* **46**, 119 (1953).
[102] W. E. J. Phillips, W. A. Maw, and R. H. Common, *Can. J. Zool.* **31**, 167 (1953).
[103] J. Fautrez and J. Moerman, *Compt. rend. assoc. anat.* **80**, 554, (1954).
[104] J. Govaert, *Compt. rend. soc. biol.* **147**, 1494 (1953).

cells of *Fasciola hepatica*, reported that the DNA content of the nucleus was more variable during intense metabolic activity than in the quiescent state. In an extensive study of nuclear size and nuclear content of DNA on various tissues of male, female and worker honeybees, Meria and Ris[105] report a high degree of polysomaty in these tissues; they found a rough correlation between the degree of ploidy and the secretory activity of the cells.

On the other hand, Leuchtenberger *et al.*[106] studying cytophotometrically the nuclei of dwarf mice with a recessive hereditary anterior pituitary hypoplasia, found a lack of the multiple DNA classes which are normally present in certain tissues of rodents and other mammals. Treatment of such dwarf mice with anterior pituitary growth hormone restored the DNA classes completely.

Pathological Tissues. In pathological conditions the DNA content of the nucleus is sometimes affected. White *et al.*[107] in an extensive chemical study of bone marrow cells with special reference to pernicious anemia found that the average value for the DNA content of marrow cells differs significantly from the normal only in the megaloblastic anemias; the possibility of polyteny is considered in this case. Bader[108] in four types of tumors found higher values of DNA distributed in classes and attributed this fact to mitosis and polyploidy. Kasten,[109] on the other hand, reports that four strains of mice differing in susceptibility to mammary cancer have the same DNA content per nucleus in the adrenal cortex, but two showed slight differences. In experimental tumors in rat liver Thomson and Frazer[98] found a higher proportion of class I nuclei (diploid), while regenerating liver contained an increased proportion of class III nuclei. In an extensive study of malignant human tissues, Leuchtenberger *et al.*[110] showed a certain deviation in the DNA content of the nucleus interpreted on the basis of mitotic activity.

In the nuclei of rectal polyps, Leuchtenberger[111] found an amount of DNA higher than the characteristic amount of normal nuclei. This seems to be in correlation with the occurence of a great number of mitotic figures. In senile keratosis, Leuchtenberger and Lund[112] found that, in the typical lesion, the content of DNA in the nuclei varied indiscriminately from normal to very high values. Pasteels and Bullough[113] also found an increase

[105] M. R. N. Meria and H. Ris, *Chromosoma* **6**, 522 (1954).
[106] C. Leuchtenberger, H. F. Helweg-Larsen, and L. Murmanis, *Lab. Invest.* **3**, 245 (1954).
[107] J. C. White, I. Leslie, and J. N. Davidson, *J. Pathol. Bacteriol.* **66**, 291 (1953).
[108] S. Bader, *Proc. Soc. Exptl. Biol. Med.* **82**, 312 (1953).
[109] F. H. Kasten, *Records Genet. Soc. Amer.* **22**, 81 (1953).
[110] C. Leuchtenberger, R. Leuchtenberger, and A. M. Davis, *Am. J. Pathol.* **30**, 65 (1954).
[111] C. Leuchtenberger, *Lab. Invest.* **3**, 132 (1954).
[112] C. Leuchtenberger, and H. Z. Lund, *Cancer Research* **12**, 278 (1952).
[113] J. Pasteels and W. S. Bullough, *Arch. biol. (Liége)* **64**, 271 (1953).

in the DNA content of epidermal nuclei under the action of croton oil. Some abnormalities reported by Leuchtenberger *et al.*[114] in the DNA content of certain samples of human sperm are probably related to sterility. Leuchtenberger *et al.*[115] studying the DNA content of spermatozoa of a large number of men (6,000 cases) showed that while fertile men had always the same characteristic amount of DNA in the spermatozoa, infertile men had significantly lower DNA values.

Dividing Cells, Mitosis, Meiosis. Pasteels and Lison[42] had considered that nuclei have a higher DNA content in dividing than in nondividing tissues and this increase could not be explained by the occurrence of mitosis. Alfert and Swift[99] demonstrated that in chick fibroblasts in tissue culture the nuclei in the migrating zone are flattened while in the dividing zone they are spherical. Because of this difference in shape, the calculations of Pasteels and Lison give values for the nuclei of the dividing zone which are 50% too high. If a proper correction is made for the difference in shape, the values of DNA are in good agreement in the two zones. Alfert and Swift[99] also criticize the data of Pasteels and Lison on cells of the crypts of Lieberkühn in the rat at different mitotic stages. They found that DNA synthesis occurs in interphase some time before prophase, and not in telophase. Errors due to stray light would explain the results of Pasteels and Lison. Roels[116] also showed that in thyroid cells of the rat stimulated to mitotic division by thiouracil, the synthesis of DNA occurs just before the onset of prophase. Moses and Taylor[117] reported that the synthesis of DNA occurs in *Tradescantia* prior to the evidence of duplication of the chromosomes in early meiotic prophase, late microspore interphase, and early pollen interphase in the generative nucleus, and concluded that DNA synthesis occurs in predivisional stages. Taylor and McMaster[118] studied by autoradiography the synthesis of DNA in microgametogenesis in *Lilium longiflorum*. This synthesis occurs before nuclear division. The DNA content of the nucleus during the course of gametogenesis was exactly related with the chromosome number. Finally, Alfert and Swift[99] found in the annelid *Sabelleria* amounts of DNA per nucleus in very good agreement with the values expected from the number of chromosomes in the different stages of the maturation of the eggs and of cleavage after fertilization, in contradiction to the earlier results of Pasteels and Lison. Swift and Kleinfeld[119] had found

[114] C. Leuchtenberger, F. Schrader, D. R. Weir, and D. P. Gentile, *Chromosoma* **6,** 61, (1953).

[115] C. Leuchtenberger, D. R. Weir, F. Schrader, and R. Leuchtenberger, *Excerpta Med. Sect. I* **8,** 418 (1954).

[116] H. Roels, *Nature* **173,** 1039 (1954).

[117] M. J. Moses and J. H. Taylor, *Records Genet. Soc. Amer.* **22,** 88 (1953).

[118] J. Herbert Taylor and R. D. McMaster, *Chromosoma* **6,** 489 (1954).

[119] H. Swift and R. Kleinfeld, *Physiol. Zool.* **26,** 301 (1953).

similar results in maturation and cleavage in the grasshopper. McMaster[120] studied sea urchin cleavage and his results, in disagreement with those of Pasteels and Lison on similar material are in agreement with the hypothesis of the constancy of the DNA in nuclei. Elson *et al.*[121] showed that the sea urchin embryo reaches a normal diploid value per cell within a few hours after fertilization, and this value remains unchanged thereafter. Marshak and Marshak[122] using the method of isotope dilution (C^{14}-labeled thymine) determined the DNA content of *Arbacia* eggs to be 8.1×10^{-6} μg. (i.e. 8.1 pg.) per egg and 7.9×10^{-7} μg (i.e. 7.9 pg.) per sperm.

Microorganisms. Ogur *et al.*[123] report the DNA content of the yeast cell as 0.0062 pg., and Webb[124] gives for the average DNA phosphorus content of the "unit cell" of *Clostridium welchii* (normal rod-shaped bacteria and filamentous forms obtained by incubation in a magnesium-deficient peptone medium) 0.00232 pg. DNA phosphorus (0.0245 pg. DNA).

[120] R. McMaster, *Anat. Record* **113,** 26 (1952).
[121] D. Elson, T. Gustafson, and E. Chargaff, *J. Biol. Chem.* **209,** 285 (1954).
[122] A. Marshak and C. Marshak, *Exptl. Cell Research* **5,** 288 (1953).
[123] M. Ogur, S. Minckler, and D. O. McClary, *J. Bacteriol.* **66,** 642 (1953).
[124] M. Webb, *Science* **118,** 607 (1953).

CHAPTER 20

Nucleic Acids in Chromosomes and Mitotic Division

BO THORELL

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I. Introduction

Reproduction in the cell is in general effected by division in which the essential process involves an equal distribution of the hereditary material between the daughter cells. The hereditary material (the genes), which forms the basis of cellular development and function, is in higher organisms mainly located to the nucleus of the cell. Nuclear division may thus be regarded as the characteristic process during cellular reproduction.

The ordinary mechanism of nuclear division is mitosis, in which the nucleus is transformed into longitudinally split chromosomes. The longitudinal halves are then distributed equally between the two daughter nuclei by a special mechanism, the spindle. This is formed at the beginning of mitosis in connection with the centriole bodies in the cytoplasm.

The chromosomes in the original sense (Flemming, Hertwig, Weissman, and others (1875–81) refer to the condensed, stainable nuclear material during mitosis.

During the stage between two successive divisions (interphase), most of the chromosomal material is dissolved into elementary structural units, also called the chromatin threads (chromonemata). In this state the nuclear material is believed to be able to perform its manifold physiological functions.

This activity seems to cease as soon as the formation of the mitotic

chromosomes starts. The thin and twisted chromatin threads of the interphase nucleus are spiralized and condensed into a small volume. The mitotic chromosomes are at the disposal of the nuclear division; they are relatively short and rigid structures and thus easily separable.

In 1868–72 Miescher[1] isolated nucleic acid from leucocytes and sperm. The similarity in composition of sperm and pus nuclei led him to foresee the importance of this substance: ". . .the study of the sperm would have a far-reaching significance for the problem of heredity." Miescher's nucleic acid was later identified as deoxypentose nucleic acid (DNA). By means of the Feulgen nucleal reaction used as a cytological staining procedure (Chapter 17), it was found[2] that the DNA present in the cell was mainly located in the chromosomal material and DNA has long been regarded as the characteristic substance of the chromosomes. Its cell-physiological function is still obscure, but collected genetical, biochemical, and cytological evidence indicates that it plays an essential part in the maintenance of heredity.

The following review gives the main results appearing from biochemical and cytological studies of the chromosomal nucleic acids and their behavior in the interphase nucleus and during mitosis. The chapter is for the sake of simplicity divided according to the different methods of investigation used. In judging the results obtained in this rather intricate field of cytochemistry, the validity of the methods used plays a dominant role. Each section, therefore, starts with a short methodological discussion.

II. The Chromosomal Nucleic Acids

1. Analyses of Isolated Chromosomes

The earlier investigations on the isolation and analysis of deoxypentose nucleic acid (DNA) were performed on whole nuclei, cells, or organs. Subsequently,[3,4] chromatin threads, "chromosomes," were isolated in amounts which permitted quantitative chemical analysis. The isolation procedure involves disruption of cell nuclei by such different means as the Waring Blendor or the colloid mill. The significance of the analytical results for the composition of the chromosomes naturally depends on how close the isolated material corresponds to the definition of chromosomes. Unfortunately, most of the classical cytological material in which the morphological details of the chromosomes can be beautifully demonstrated does not lend itself to isolation in bulk. Instead, nuclei from mammalian thymus or liver or from fish or bird erythrocytes have been used.

[1] F. Miescher, "Die histochemische und physiologische Arbeiten." Leipzig, 1897.
[2] R. Feulgen and H. Rossenbeck, *Z. physiol. Chem.* **135**, 203 (1924); R. Feulgen, *Ber. Physiol.* **22**, 489 (1924).
[3] A. E. Mirsky and A. W. Pollister, *Biol. Symposia* **10**, 243 (1943).
[4] A. Claude and J. S. Potter, *J. Exptl. Med.* **77**, 345 (1943).

Mirsky, Pollister, and Ris[3,5] carried out painstaking morphological studies to prove that the threadlike structures isolated by them in fact were chromosomes from the interphase nuclei. Their conclusions have, however, been disputed[6] by the claim that the threads are not preformed structures in the nucleus but artifacts, produced by drawing out the more or less fragmented nuclei during the treatment.

Denues,[7] in a series of publications, has scrutinized the different phases of the isolation procedure with the aid of the electron microscope. Many characteristic features, such as a certain differentiation along the axis of the threads, coiled structures, and occasional visible doubling, are cited as evidence for the chromosome nature of the threads. A certain common pattern can also in many cases be found in threads isolated from different organs of the same species. The morphological evidence that the chromatin threads isolated are true chromosomes of interphase nuclei has been summarized by Ris and Mirsky (1951).[8] The threads constitute a morphological fraction which can be isolated readily, and much of it can no doubt be identified as interphase chromosomes or chromosome pairs.[9]

In any consideration of the identity of the structures isolated, it must be kept in mind that the process of isolation may involve contamination of the particles or extraction of material from them. Such contamination or loss may seriously affect the nucleic acid composition of isolated chromosomes. In some material, such as bird erythrocytes, cytoplasmic contamination will add very little to the nucleic acid figures. On the other hand, in tissues such as liver the apparent PNA content of isolated chromosomes will be substantially increased if they are contaminated with cytoplasmic particles. The same risk also holds for isolated nuclei.[10] Therefore, an adequate morphological control preferably by phase-contrast microscopy (or electron microscopy) during the isolation procedure is essential. Isolation in widely differing media, e.g., those used by Stern and Mirsky,[11] can give information about undesirable extraction conditions.

About 90 % of the mass of isolated chromosomes can be extracted with 1 *M* NaCl.[12] The extracted material consists mainly of nucleohistone, of which 45 % is deoxyribonucleic acid and 55 % is low-molecular-weight protein of the histone type. The insoluble residue after NaCl extraction still shows threadlike structures ("residual chromosomes") which are, however,

[5] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **31,** 1 (1947); **34,** 475 (1951).
[6] W. G. P. Lamb, *Nature* **164,** 109 (1949); *Exptl. Cell Research* **1,** 571 (1950).
[7] A. R. T. Denues, *Exptl. Cell Research* **3,** 540 (1952); **4,** 333 (1953).
[8] H. Ris and A. E. Mirsky, *Exptl. Cell Research* **2,** 263 (1951).
[9] A. W. Pollister, *Exptl. Cell Research* Suppl. 2, 73 (1952).
[10] R. Y. Thomson, F. C. Heagy, W. C. Hutchinson, and J. N. Davidson, *Biochem. J.* **53,** 460 (1953).
[11] H. Stern and A. E. Mirsky, *J. Gen. Physiol.* **37,** 177 (1953).
[12] A. E. Mirsky and H. Ris, *J. Gen. Physiol.* **31,** 7 (1947); *Nature* **163,** 666 (1949)

reduced in size as compared with the original isolated chromosomes. They consist of 80% high-molecular-weight protein containing *inter alia* 1.36% tryptophan and resembling the "chromosomin" isolated from nuclei by Stedman and Stedman.[13] This may perhaps represent the structural backbone of the chromosomes which is of importance for the cytogenetical phenomenon of breakage induced, e.g., by radiation.[14] The concept of a structural function is consistent with the trypsin digestion experiments mentioned later in Section II.2.*b*. The PNA content of the residual chromosomes varies between 7.5 to 14%. Some DNA is also present (1.5 to 2.6%). Compared with the whole isolated chromosomes, the nucleic acid content of the "residual chromosomes" is thus relatively small in amount and mainly PNA in type.

It should be noted that the proportions of the various constituents analyzed as mentioned above seem to vary in isolated chromosomes from different tissues. A larger proportion (40 to 50%) of chromosomes from liver consists of "residual chromosomes" as compared with chromosomes from erythrocyte nuclei (5%). Consequently liver chromosomes contain more PNA (12%) than thymus (3%) or trout sperm (0.15%) chromosomes. Probably this reflects the different functional states of the respective nuclear material. Changes in the DNA content of isolated chromosomes from different stages during experimental carcinogenesis have been reported.[15]

It is generally believed that all of the highly polymerized DNA of the cell is contained in the chromosomes and that each complete set of chromosomes contains a constant amount of DNA (see Chapter 19). On the other hand, if the analytical values for PNA from isolated chromosomes are compared with those obtained from isolated nuclei,[10] it will be seen that in liver nuclei there must be some extrachromosomal PNA provided that no PNA is lost during the preparation procedure. A part of this is probably bound to the nucleolus, the morphology of which varies considerably with different functional states. The physiological importance of this observation is still however obscure. The nucleic acids of the nucleolus are discussed in Chapter 18.

Summarizing, it may be said that the analysis of isolated chromosomes has given a picture of the main quantitative composition of these structures, but the informations obtained must be regarded with caution owing to the general pitfalls associated with the isolation in bulk of a particular cellular structure.

[13] E. Stedman and E. Stedman, *Nature* **152**, 267 (1943); *Cold Spring Harbor Symposia Quant. Biol.* **12**, 224 (1947).

[14] A. H. Sparrow, M. J. Moses, and R. J. DuBow, *Exptl. Cell. Research* Suppl. 2, 245 (1952).

[15] A. R. Gopal-Ayengar and E. V. Cowdry, *Cancer Research* **7**, 1 (1947).

2. Analysis of Chromosomes *in Situ*

a. The Feulgen Reaction

The chromosomes can be visualized in situ by their ability to combine with specific dyes. Since the work of Feulgen and Rossenbeck,[2] the method most commonly utilized for demonstrating the chromosomal DNA has been the Feulgen nucleal reaction, which is discussed in detail in Chapter 17.

The exact course of the Feulgen reaction is not yet fully understood, but it is generally assumed that a liberation of aldehyde groups from the DNA takes place through partial acid hydrolysis and that these aldehyde groups react with leuco-basic fuchsin (the Schiff reagent) forming a red-colored product. This reaction has also been used for the microchemical determination of DNA in amounts between 0.5 and 2 mg. but not in the presence of proteins.[16,17] Since the reaction does not proceed stoichiometrically, a DNA standard always must be used at the same time.

Nevertheless, the Feulgen nucleal reaction has been used by several authors for the quantitative determination of DNA in histological preparations.[18] Since, in nuclei with a DNA concentration up to a few per cent, the intensity of the color developed is proportional to the DNA content,[19] the relative amounts of DNA per nucleus in a cell population can in some cases be estimated,[20-23] and the results expressed in arbitrary units.

There has been considerable controversy concerning the specificity of the Feulgen reaction in cytological preparations, but it is generally agreed at the present time that common biological materials contain little or no material other than DNA that can give a proper nucleal reaction. Whether DNA, with or without the recolorized leuco-basic fuchsin, is able to diffuse throughout the cell so as to give a false picture of localization has also been discussed.[24,25] Partially hydrolyzed DNA from which only purine bases have been split off,[26] is however insoluble in weak acid and reacts with the Schiff reagent to give the characteristic bluish-red color. This color is developed on the insoluble material, the solution above it remaining colorless.

Thus the most important conclusion from the numerous investigations carried out by means of the Feulgen nuclear reaction can be regarded as valid, namely, that the DNA in the cell is in general concentrated in the chromosomes. This conclusion holds for material after acid hydrolysis and

[16] G. Widström, *Biochem. Z.* **199,** 298 (1928).
[17] T. Caspersson, *Biochem. Z.* **253,** 97 (1932).
[18] H. S. di Stefano, *Chromosoma* **3,** 282 (1948).
[19] H. Ris and A. Mirsky, *J. Gen. Physiol.* **33,** 125 (1949).
[20] R. E. Stowell, *J. Natl. Cancer Inst.* **3,** 111 (1942).
[21] A. W. Pollister and H. Ris, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 147 (1947).
[22] H. H. Swift, *Physiol. Zool.* **23,** 169 (1950).
[23] C. Vendrely, *Bull. biol. France et Belg.* **84,** 1 (1952); see also A Pollister, M. Himes, and L. Ornstein, *Federation Proc.* **10,** 629 (1951).
[24] E. Stedman and Ellen Stedman, *Symposia Soc. Exptl. Biol.* **1,** 232 (1947).
[25] H. N. Barber and H. G. Callan, *Nature* **163,** 109 (1949); see also J. O. Ely, and M. H. Ross, *Anat. Record* **194,** 103 (1949).
[26] C. H. Li and M. Stacey, *Nature* **163,** 538 (1949); W. G. Overend and M. Stacey, *ibid.* **163,** 538 (1949).

usually after treatment with histological fixatives since such treatment in some special cases can cause a redistribution of DNA within the cell.[27]

In suitable material some details of localization of DNA in the chromosomes can be studied by the Feulgen reaction. The giant chromosomes in salivary gland cells of *Drosophila* are regarded as bundles of extended, similar chromonemata lying side by side.[28-30] Each chromonema carries Feulgen-positive granules of various sizes. The corresponding granules of adjacent chromonemata are arranged across the width of the chromosomes in the form of bands. These granules have been claimed to represent the gene loci.[31,32]

The threadlike structures isolated from interphase nuclei and considered to be chromosomes (see Sect. II.1) also show a positive Feulgen reaction[4] with, in many cases, beadlike formations along paired filaments.[5,8] The different pictures of the various types of interphase nuclei after staining with the Feulgen nucleal reagent can be explained by the different mode of packing of the chromosome threads. Furthermore, in many cell types, the Feulgen-positive material (DNA) of the chromosomes tends to coalesce forming a chromocentrum, the functional significance of which is unknown.

During mitosis the chromosomes can usually be beautifully demonstrated in situ by staining the DNA with the Feulgen procedure.[33,34] The structural changes mentioned in the introduction can thus be followed in the essential details (see also Sect. III).

b. Staining Methods

Investigations of nuclear substances with acid and basic dyes date from the work of Fleming (1875–81),[35] who defined chromatin as a substance showing strong affinity for dyestuffs and stated: "Es ist möglich, dass diese Substanz geradezu identisch ist mit den Nukleinkörpern. . . ." It was shown by several workers that staining was dependent more or less on the charge on the cellular substances. Malfatti (1892)[36] used mixtures of acid fuchsin and the basic dye methyl green. With nucleic acid and protein compounds

[27] J. Chayen and K. P. Norris, *Nature* **171,** 472 (1953).
[28] E. Heitz and H. Bauer, *Z. Zellforsch.* **17,** 67 (1933).
[29] E. Heitz, *Z. indukt. Abstamm.-u. Vererblehre* **67,** 216 (1934); see also H. Bauer, *Zool. Jahrb. Physiol.* **56,** 239 (1936).
[30] T. S. Painter, *J. Heredity* **25,** 465 (1934).
[31] T. S. Painter, *Genetics* **19,** 175 (1934).
[32] C. B. Bridges, *Am. Naturalist* **56,** 51 (1922); see also T. H. Morgan, C. B. Bridges, and J. Schultz, *Carnegie Inst. Wash. Yearbook* **33,** 274 (1934).
[33] H. Voss, *Z. Mikroskop-anat. Forsch.* **33,** 222 (1933).
[34] A. Hughes, "The Mitotic Cycle." Butterworth, London, Academic Press, New York, 1952.
[35] W. Flemming, "Zellsubstanz, Kern und Zellteilung." Leipzig, 1882.
[36] H. Malfatti, *Z. physiol. Chem.* **16,** 68 (1892).

prepared according to Altmann,[37] he showed the affinity of nucleic acid for methyl green and of protein for the acid dye. The relationship between the dye and the cellular substances was also studied[38,39] in its qualitative and quantitative aspects.[40-42] The competitive action of protein on the formation of the dye-nucleate salt was shown to be such that a stoichiometric relationship between the amount of dye bound to a cellular structure and its content of nucleic acid could scarcely be expected, particularly since adsorption of dye on the cell surfaces and its solubility in the cellular structures also have to be considered.

Nevertheless, several papers on the estimation of nucleic acid in the nucleus using quantitative dye measurements have been published. In some cases under strictly standardized conditions a fairly constant relationship has been obtained between the amounts of bound dye in a cytological preparation and the nucleic acid content determined according to other methods.[43-46] These questions are discussed in detail in Chapter 17.

While methyl green staining shows a certain specificity as regards the chromosomal DNA, the selective demonstration of PNA with basic dyes is doubtful. Pyronin has been extensively used but cannot be regarded as specific.[47]

The isolation and purification of specific enzymes attacking the two types of nucleic acid[48] has given new opportunities for studying the distribution of DNA and PNA in the chromosomes although the conditions essential in the application of cytochemical methods employing enzymic hydrolysis must be carefully controlled. Not only must the purity of the enzyme and the absence of proteolytic contaminants be assured, but all other variables capable of modifying the reactions must be examined, e.g., fixation and embedding procedures, pH, time and temperature of digestion, etc.

On the basis of altered stainability effected by enzymic treatment, the presence in chromosomes of both DNA and PNA has been demonstrated.[49-53]

[37] R. Altmann, *Arch. Anat. u. Physiol.* **13,** 524 (1889).
[38] K. Spiro, "Über physikalische und physiologische Selection." Strassburg, 1897.
[39] A. Mathews, *Am. J. Physiol.* **1,** 445 (1898); see also M. Heidenhain, *Pflügers Arch. ges. Physiol.* **90,** 115 (1902).
[40] L. Michaelis and P. Rona, *Biochem. Z.* **97,** 57 (1919).
[41] J. Loeb, "Proteins and the Theory of Colloidal Behaviour." McGraw Hill, New York, 1922.
[42] E. Hammarsten, G. Hammarsten, and T. Teorell, *Acta Med. Scand.* **58,** 219 (1928).
[43] A. Mirsky, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 143 (1947).
[44] N. Kurnick, *J. Gen. Physiol.* **33,** 243 (1950); *Exptl. Cell Research* **1,** 151 (1950).
[45] N. Kurnick and A. Mirsky, *J. Gen. Physiol.* **33,** 265 (1950).
[46] L. Michaelis, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 131 (1947).
[47] E. Taft, *Exptl. Cell Research* **2,** 312 (1951).
[48] J. H. Northrop, M. Kunitz, and R. M. Herriott, "Crystalline Enzymes," 2nd ed. Columbia Univ. Press, New York, 1948.
[49] J. Brachet, *Compt. rend. soc. biol.* **133,** 88 (1940).
[50] J. Schultz, *Cold Spring Harbor Symposia Quant. Biol.* **9,** 55 (1941).

The results correspond reasonably well with those obtained by analysis of isolated chromosomes (see Sect. II.1). Thus, by using ribonuclease in combination with an acid dye which stains protein but not PNA, the association of PNA with protein in the chromosomes is confirmed.[51,54] The action of deoxyribonuclease on chromosomes appears to release some protein material both of the histone and nonhistone type. Degradation of one material could thus also result in the disappearance from the chromosomes of other associated substances.

Digestion of the chromosomal PNA in a cytological preparation seems to affect the DNA in such a way that its affinity for methyl green is reduced.[51,55] If contamination with deoxyribonuclease can be excluded, this observation suggests an association *in situ* between PNA and DNA which may be of importance in relation to the state of nucleic acid polymerization.[56]

The removal of the nucleic acids from the chromosomes by enzymes or by extraction with trichloroacetic acid leaves a residue of protein, stainable with acid dyes.[51] The principal threadlike structure still remains. If these proteins are hydrolyzed by trypsin, the chromosomal structure disintegrates. This occurs whether the polymerized nucleic acids are intact or precipitated before the digestion of protein.[57,58]

The experimental results obtained by the use of dyes, especially in combination with specific enzymic hydrolysis, have thus given a fairly intricate pattern of association between the different chromosomal substances, a pattern which at present cannot very easily be physiologically interpreted.

Moreover from a purely methodological point of view, many problems remain to be solved, such as the interaction of the different chromosomal substances and its influence on substrate specificity, and the activation and inhibition of the enzymic hydrolysis of an intact cellular structure.

c. Radiation Absorption Measurements

As a result of their purine and pyrimidine content, the nucleic acids have a specific absorption in the ultraviolet region around 260 mμ.[59,60] This is discussed in detail in Chapter 14. Their absorption constants make them detectable within the ranges of concentration and layer thicknesses in which

[51] B. P. Kaufmann, M. R. McDonald, and H. Gay, *J. Cellular Comp. Physiol.* **38,** Suppl. 1, 71 (1951).

[52] B. P. Kaufmann, M. R. McDonald, H. Gay, K. Wilson, and R. Wyman, *Carnegie Inst. Wash. Yearbook* **46,** 141 (1947).

[53] B. P. Kaufmann, *Science* **109,** 443 (1949).

[54] B. P. Kaufmann, M. R. McDonald, and H. Gay, *Am. J. Botany* **38,** 268 (1951).

[55] M. R. McDonald, *J. Cellular Comp. Physiol.* **38,** Suppl. 1 (Discussion), 89 (1951).

[56] A. Mirsky, *J. Cellular Comp. Physiol.* **38,** Suppl. 1 (Discussion), 92 (1951).

[57] T. Caspersson, E. Hammarsten, and H. Hammarsten, *Trans. Faraday Soc.* **31,** 367 (1935).

[58] T. Caspersson, *Skand. Arch. Physiol.* **73,** Suppl. 8 (1936).

[59] C. Dhéré, *Compt. rend. soc. Biol.* **60,** 34 (1906).

[60] F. Heyroth and J. Loofbourow, *J. Am. Chem. Soc.* **53,** 3341 (1931); see also *J. Am. Soc.* **56,** 1728 (1934).

they occur in many cells. Other cellular substances containing the chromophoric pyrimidine group show a similar high absorption at 260 mμ, e.g., nucleosides and nucleotides. By comparison with the cellular proteins the specific absorption of nucleic acids is much higher (about 100 times), and only those proteins containing the aromatic amino acids have a specific absorption in this wavelength region.[58,61]

The high absorption of the nucleic acids has been used to investigate by microoptical methods their distribution and content in the chromosomes both in the resting nucleus and during the mitotic cycle.

Absorption in the regions of soft X-rays[62,63] and infrared mainly between 3 and 15 μ[64] has also been utilized in the same way but to a much lesser extent than ultraviolet absorption.

The first ultraviolet microscope was constructed about 1900[65,66] primarily to gain a higher resolving power than was available with the ordinary light microscope. By using the new microscope Köhler (1904)[65] and von Schrötter (1906)[67] noted the richness in detail exhibited by various types of cells, both living and fixed. With regard to the high ultraviolet absorption in the chromatin and especially in the chromosomes (Köhler[65]), von Schrötter[67] made the assumption "dass die Absorption für ultraviolettes Licht mit dem Nucleingehalte des Gewebes oder der einzelnen Strukturen zunimmt." The "Nuclein" had previously been described by Miescher[1] and Kossel.[68]

In spite of the fact that ultraviolet microscopy was suggested several times as a tool for cytochemical analysis,[65,69,70] it was during the next three decades mainly used only for structural studies on living or unstained material. It is true that some theoretical considerations and some measurements were published,[69,71-73] but no attempt was made to solve the obvious difficulties of introducing absorption measurements in the ultraviolet microscope as an analytical technique. Interesting pictures, however, were shown in the micrographs of living chromosomes in grasshopper spermatocytes by Lucas and Stark[74] and the mitotic figures in tissue culture cells by

[61] E. R. Holiday, *Biochem. J.* **24,** 619 (1930).
[62] A. Engström and B. Lindström, *Biochem. et Biophys. Acta* **4,** 351 (1950).
[63] A. Engström and F. Ruch, *Proc. Natl. Acad. Sci. U.S.* **37,** 459 (1951).
[64] R. Frazer and J. Chayen, *Exptl. Cell Research* **3,** 492 (1952).
[65] A. Köhler, *Z. wiss. Mikroskop.* **21,** 129 (1904).
[66] A. Köhler and M. von Rohr, *Z. Instrumentenk.* **24,** 341 (1904).
[67] H. von Schrötter, *Virchow's Arch. pathol. Anat. u. Physiol.* **183,** 343 (1906).
[68] A. Kossel, *Z. physiol. Chem.* **7,** 7 (1882).
[69] A. Köhler and A. Tobgy, *Arch. Augenheilk.* **99,** 263 (1928).
[70] R. W. G. Wyckoff and A. H. Ebeling, *J. Morphol.* **55,** 131 (1933).
[71] W. Swann and C. del Rosario, *J. Franklin Inst.* **213,** 549 (1932).
[72] F. F. Lucas, *J. Franklin Inst.* **217,** 661 (1934).
[73] F. Vlès and M. Gex, *Arch. phys. biol.* **11,** 157 (1934).
[74] F. F. Lucas and M. B. Stark, *J. Morphol.* **52,** 91 (1931).

Wyckoff *et al.*,[75] both confirming the earlier observations of Köhler and von Schrötter. Wyckoff *et al.* also commented on the similarity between the ultraviolet photomicrographs and those of Feulgen-stained preparations.

The ultraviolet absorption of the chromosomal nucleic acids was discussed in a paper by Caspersson, E. and H. Hammarsten[57] in 1935, in which they showed that chromosomes from *Stenobotrus* testicular cells did not change their absorption at 280 mμ after digestion with trypsin-lanthanum reagent. Similar results were obtained on the bands of *Drosophila* giant chromosomes, and the authors concluded that these structures contained large amounts of nucleic acids.

From this time onwards many cell-physiological problems concerning the nucleic acids were studied with the help of ultraviolet microscopy by the Stockholm group[76] and also by a group at King's College, London.[77] It has been made quite clear that the technique has important biological applications. But theoretical as well as practical experience has shown that the optical and physicochemical properties of the common cytological material, and also the optical conditions in the microscope, are factors not yet completely understood. Consequently, the quantitative analytical application of ultraviolet microscopy is somewhat complicated if valid results are to be obtained. Recent discussions of these questions can be found in papers by Glick *et al.*[78] and Davies and Walker.[79] The analytical procedure also involves the necessity for strictly defined conditions as regards technical equipment, and references are here made to some pertinent papers.[80-86] This question is also discussed in some detail in Chapter 17.

For chromosomal analysis with ultraviolet microspectrography, the giant chromosomes from *Drosophila* larvae have frequently been used. From a topographical point of view, these chromosomes have certain advantages since they represent very much enlarged nuclear structures of haploid type formed by endomitotic division and pairing. The different parts of the chromosme consisting of euchromatin, heterochromatin, certain gene loci, etc., can with certainty be cytologically defined.[28-32] Analysis

[75] R. W. G. Wyckoff, A. H. Ebeling, and A. L. Ter Louw, *J. Morphol.* **53,** 189 (1932).
[76] T. Caspersson, "Cell Growth and Cell Function." Norton, New York, 1950.
[77] see J. T. Randall, *Discussions Faraday Soc.* **9,** 353 (1950).
[78] D. Glick, A. Engström, and B. G. Malmström, *Science* **114,** 253 (1951).
[79] H. G. Davies and P. M. B. Walker, *Progr. Biophys. and Biophys. Chem.* **3,** 195 (1953).
[80] T. Caspersson, *J. Roy. Microscop. Soc.* **60,** 8 (1940).
[81] B. Thorell, "Studies on the Formation of Cellular Substances during Blood Cell Formation." Henry Kimpton, London, 1947.
[82] B. Thorell, *Discussions Faraday Soc.* **9,** 432 (1950).
[83] M. Wilkins, *Discussions Faraday Soc.* **9,** 363 (1950).
[84] R. Barer, E. Holiday, and E. M. Jope, *Biochim. et Biophys. Acta* **6,** 123 (1950).
[85] R. Mellors, *Discussions Faraday Soc.* **9,** 398 (1950).
[86] T. Caspersson, F. Jacobsson, and G. Lomakka, *Exptl. Cell. Research* **2,** 301 (1951).

of these structures shows high ultraviolet absorption of the chromosome bands with a maximum at 260 mμ.[87-89] On the other hand, the ultraviolet absorption of the interband spaces was very low and shows a maximum at 280 mμ. On the basis of these findings, Caspersson[76,88,89] estimated that the nucleic acid content in the bands was as high as 10 to 30%. The interband spaces consisted only of protein containing tyrosine and tryptophan in concentrations of 5% and 2%, respectively.[76,88,89]

A correction of this interpretation, however, was later made by Engström and Ruch,[63] who showed by X-ray microradiography that, in fact, most of the chromosomal mass is localized in the bands and that the interband spaces are almost empty. As a result of their high content of organic substance, the bands can be expected to absorb ultraviolet light to a high extent, both specifically and unspecifically.

The ultraviolet-absorbing substances in the division chromosomes from the salamander can be almost completely extracted by hot trichloroacetic acid. Since the spindle fibers and asters also lose their ultraviolet absorption after extraction, Pollister and Ris[90,91] have concluded that both chromosomes, spindle fibers, and asters contain substantial amounts of nucleic acids. Walker, Davies, and Yates,[92-94] in extensive studies on tissue culture cells, confirmed the specific absorption at 260 mμ of the interphase and mitotic nuclei. In presenting their results they state with caution that the amount of nitrogenous bases has been calculated from the ultraviolet absorption "as if they were combined as nucleic acid, without prejudice to their actual conformations." They find that the absorption spectra in their material are, in general, of nucleoprotein type and that the aromatic amino acid contribution near the nucleic acid peak is very small, like the apparent (unspecific) absorption at 312 mμ. Their results are concerned with changes in the "nucleic acid absorption" during growth and division of the tissue culture cells and will be referred to in greater detail in Section III.

In this connection some cytogenetical experiments performed on *Drosophila* giant chromosomes by Schultz in collaboration with Caspersson may be noted.[95-98] There is in *Drosophila melanogaster* a group of chromosome

[87] T. Caspersson, *Naturwissenschaften* **28**, 514 (1940).
[88] T. Caspersson, *Chromosoma* **1**, 605 (1940).
[89] T. Caspersson, *Naturwissenschaften* **29**, 29 (1941).
[90] A. Pollister and H. Ris, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 147 (1947).
[91] H. Ris, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 158 (1947).
[92] P. M. B. Walker and H. G. Davies, *Discussions Faraday Soc.* **9**, 461 (1950).
[93] P. M. B. Walker and H. B. Yates, *Symposia Soc. Exptl. Biol.* **6**, 265 (1952).
[94] P. M. B. Walker and H. B. Yates, *Proc. Roy. Soc.* (*London*) **B140**, 274 (1952).
[95] J. Schultz and T. Caspersson, *Arch. exptl. Zellforsch. Gewebezücht.* **22**, 650 (1939).
[96] J. Schultz, *Cold Spring Harbor Symposia Quant. Biol.* **9**, 55 (1941).
[97] J. Schultz, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 179 (1947).
[98] T. Caspersson and J. Schultz, *Nature* **142**, 294 (1938).

rearrangements which causes the individual carrying them to be variegated for characters associated with genes located at one of the rearrangement points. The other point of rearrangement is in one of the heterochromatic regions. If the bands of the gene loci in question in a normal chromosome show values for ultraviolet absorption at 260 mμ of about 6 %, the same bands translocated close to a heterochromatic region absorb about 20 %. This may indicate that, like the genetically known position effect, the ultraviolet absorption of a chromosome band, presumably to some extent conditioned by its nucleic acid content, is likewise dependent on its position in the chromosome.

The orientation of the nucleic acids in the chromosomes has been the object of discussion on the basis of the high dichroic ratio shown by polymerized DNA.[99,100] Oriented DNA films show dichroic ratios up to 4.7 depending on the humidity.[101,102] Except in certain materials, such as sperm heads, the orientation of the nucleic acids giving a measurable dichroism is very low in cytological material including *Drosophila* chromosomes[103]. Hence it is technically difficult to estimate. No results have so far appeared on this important structural problem, but special instruments have been designed.[104]

The use of the ultraviolet microscope has offered unique possibilities for the localization of nucleic acids in the cell. But it has become generally recognized that the interpretation of the ultraviolet absorption spectra is not at all unequivocal. In the chromosomes there are certainly ultraviolet-absorbing substances other than DNA but quantitatively as important as the DNA. For example, a great proportion of PNA must be considered. As the quantitative microoptical determinations can now be performed with a high precision, it is also necessary to consider how far the absorbing substances correspond with the chemically defined compounds (polymerized nucleic acids, nucleotides, other aromatic compounds, unsaturated fatty acids, etc.). In this respect, complementary information can be obtained by infrared microspectroscopy, as mentioned above, and also by studies involving digestion with specific enzymes under the proper conditions.

III. Changes in the Chromosomal Nucleic Acids During the Cell Cycle

1. Quantitative Changes

Two main processes connected with mitosis can be distinguished in relation to the chromosomal nucleic acids. Firstly, at some stage during the

[99] B. Commoner and D. Lipkin, *Science* **110**, 41 (1949).
[100] M. Wilkins, *Discussions Faraday Soc.* **9**, 368 (1950).
[101] B. Thorell and F. Ruch, *Nature* **167**, 815 (1951).
[102] W. Seeds, *Progr. Biophys. and Biophys. Chem.* **3**, 27 (1953).
[103] T. Caspersson, *Chromosoma* **1**, 605 (1940).
[104] F. Ruch, personal communication.

cell cycle there must be a new formation of nucleic acids in amounts equal to those in the mother nucleus (see also Chapter 19); secondly, these substances have to be distributed between the two daughter nuclei.

Since the fundamental cytological studies of Flemming[105] and Strasburger,[106] it seems to have been generally believed that the new formation of the chromosomal substances takes place in close relation to the process of mitosis itself. This view, that most of the DNA of the chromosomes is synthesized *de novo* at the beginning of each division, is still maintained by many.[76,107-109]

The attempts to analyze the new formation of the chromosomal nucleic acids and its relationship to the cell cycle have involved principally two different techniques. Quantitative analysis of the nucleic acid content of single dividing cells, as well as of cells in bulk undergoing synchronous divisions, has given information about the stage of the cell cycle during which the main increase of the nuclear nucleic acids occurs. Secondly, isotope tracers have been used to determine the time and magnitude of incorporation into the nucleic acids in relation to mitosis.

Naturally occurring synchronous divisions suitable for macrochemical analyses are rare but they can, however, be produced experimentally. After partial hepatectomy in the rat, a sharp peak in the frequency of mitosis occurs at 24 hours. As many as 10 % of the cells may undergo mitosis at the same moment. It has been found that prior to the appearance of the mitotic figures, the average DNA content of the liver nuclei had increased from 10 to 18 pg. per nucleus.[110] The synthesis of DNA begins only a few hours after partial hepatectomy[111-113] and can be demonstrated by increasing incorporation of labeled glycine into the nitrogenous bases of both DNA and nuclear PNA.[114-115]

Zeuthen *et al.*,[116,117] using intermittent heat treatment of mass cultures

[105] W. Flemming, *Arch. mikroskop. Anat. u. Entwicklungsmech.* **16**, 302 (1879).
[106] E. Strasburger, "Zellbildung und Zellteilung." Jena, 1880.
[107] C. D. Darlington, *Symposia Soc. Exptl. Biol.* **1**, 252 (1947).
[108] M. J. D. White, "Animal Cytology and Evolution." Cambridge Univ. Press, New York, 1948.
[109] C. D. Darlington and K. Mather, "The Elements of Genetics," Allen & Unwin, London, 1949.
[110] J. M. Price and A. K. Laird, *Cancer Research* **10**, 650 (1950).
[111] A. M. Brues, D. R. Drury, and M. C. Brues, *Arch. Pathol.* **22**, 658 (1936).
[112] A. M. Brues, M. M. Tracy, and W. E. Cohn, *J. Biol. Chem.* **155**, 619 (1944).
[113] H. B. Novikoff and V. R. Potter, *J. Biol. Chem.* **173**, 233 (1948).
[114] E. Hammarsten, *in* "Isotopes in Biochemistry" (J. N. Davidson, ed.), p. 203. Churchill, London, 1951.
[115] N. A. Eliasson, E. Hammarsten, P. Reichard, S. E. G. Åqvist, B. Thorell, and G. Ehrensvärd, *Acta Chem. Scand.* **5**, 431 (1951).
[116] E. Zeuthen, *J. Embryol. and Exptl. Morphol.* **1**, 239 (1953).
[117] O. Scherbaum and E. Zeuthen, *Exptl. Cell. Research* **6**, 221 (1954), and personal communication.

of *Tetrahymena*, have recently induced 85% of the cells to divide simultaneously. During the period of heat treatment growth was not inhibited; for example, the DNA content per cell increased up to four times. This material thus seems excellent for the study of the division processes as several milligrams of dry substance can easily be obtained.

The increase in the nucleic acid content of individual cells during the cycle has been studied by different cytochemical techniques. From ultraviolet absorption measurements at different stages in the spermatogenesis of *Gomphocerus*, Caspersson[118] calculated an increase in the nuclear nucleic acids from early prophase to metaphase from about 16 to 26 $\times$ 10^{-9} mg. Similar results are reported by Ris.[91] From the different shapes of the absorption curves, Caspersson calculated a change in the ratio of nucleic acids to protein from 1:20 in the early prophase chromosomes to 1:3 in the metaphase chromosomes.[76,87,88] During telophase-interphase the changes were reversed. As a result of the nature of the method and the materials used, however, the values mentioned above may be regarded more as an expression of general changes in density than as nucleic acid and protein changes.

Particularly suitable material has been studied by Walker and Yates[92-94,119,120] in the form of actively dividing tissue culture cells which were investigated directly under the ultraviolet microscope. Taking into consideration the sensitivity towards ultraviolet irradiation, the cells were observed by phase-contrast microscopy and the ultraviolet absorption was measured only at certain defined stages of the cycle. In spite of the relative unspecificity of the ultraviolet-absorption microtechnique, a fair agreement was found in certain materials between the amount of DNA calculated from the absorption at 265 mμ and values from the literature for total DNA-phosphorus in known numbers of nuclei. No sudden increase in ultraviolet-absorbing material could be demonstrated during prophase, but a continuous augmentation was observed during the interphase. When the ultraviolet absorption and the Feulgen-staining intensity were compared, the authors concluded that the actual increase in DNA occurred during interphase. The difference observed between the total nuclear ultraviolet absorption and the Feulgen color was interpreted as being due partly to the presence of precursors of nucleic acids.

Indirect evidence of DNA synthesis during interphase in growing cells has been put forward by Swift and others,[22,121-126] on the basis of microphotometric estimations on individual nuclei stained by the Feulgen reac-

[118] T. Caspersson, *Chromosoma* **1**, 147 (1939).
[119] P. Walker, *Discussions Faraday Soc.* **9**, 497 (1950).
[120] P. Walker, *Heredity* Suppl. 6, 275 (1952).
[121] H. Swift, *Anat. Record* **105**, 497 (1949).
[122] H. Swift, *Proc. Natl. Acad. Sci. U. S.* **36**, 643 (1950).

tion (see also Sect. II.2.*a*). The spread of values obtained from interphase nuclei in dividing cells was in marked contrast to the sharply defined classes found in differentiated, nongrowing tissue. Klein *et al.*[127,128] however, found no significant difference in the statistical distribution of the total ultraviolet absorption at 265 mμ in individual isolated nuclei from certain diploid ascites tumors compared with nongrowing lymphocyte and histiocyte nuclei. The doubling of the chromosomal DNA in this material thus seems to proceed during a relatively short period immediately before or after mitotic division. It may be that this behavior is peculiar to tumor cells. It should be mentioned that in this tumor material good agreement was obtained between macrochemical estimations of DNA-phosphorus, ultraviolet absorption values, and also the degree of ploidy.[129,130]

Interesting suggestions concerning the mechanism of distribution of nucleic acids during mitotic division have been made by Jacobson and Webb,[131,132] using the ordinary May-Grünwald and Giemsa stains in combination with digestion by ribo- and deoxyribonucleases. The prophase and telophase chromosomes stained red in contrast to the blue-black staining of the metaphase chromosomes. The authors concluded that DNA-proteins were present in the chromosomes of the interphase nucleus and in the division chromosomes. PNA-proteins, on the other hand, were present in the chromosomes only from the end of prophase, through meta- and anaphase, up to early telophase. During the anaphase movement, PNA-proteins appeared to be shed from the chromosomes into the space between the two groups of chromosomes. In spite of painstaking experiments to show the specificity of the May-Grünwald and Giemsa stain, these conclusions seem not fully convincing. As mentioned in Section II.2.*b* previous studies have shown the reactions between dyes and the intracellular structures to be a complicated matter of adsorption, salt linkages, etc. Moreover, the results of analyses of interphase chromosomes (Sect. II.1) make it improbable that these structures are devoid of PNA. It seems probable that Jacobson and Webb are dealing with effects of interaction between quantitative changes of DNA, PNA, and proteins, the magnitudes of which cannot be evaluated from the staining properties. All these findings need

[123] F. Schrader and C. Leuchtenberger, *Proc. Natl. Acad. Sci.* **35,** 464 (1949).
[124] J. Pasteels and L. Lison, *Arch. biol. (Liége)* **61,** 445 (1950).
[125] A. H. Sparrow, M. J. Moses and R. Steele, *Brit. J. Radiol.* **25,** 182 (1952).
[126] H. Swift, *Intern. Rev. Cytol.* **2,** 1 (1953).
[127] G. Klein and G. Moberger, Report from Gustaf V. Research Foundation, Stockholm, 1953.
[128] G. Klein, Eva Klein, and Elin Klein, *Cancer Research* **12,** 484 (1952).
[129] C. Leuchtenberger, G. Klein, and E. Klein, *Cancer Research* **12,** 480 (1952).
[130] T. Hauschka and A. Levan, *Exptl. Cell Research* **4,** 457 (1953).
[131] W. Jacobson and M. Webb, *J. Physiol.* **112,** 2P (1950).
[132] W. Jacobson and M. Webb, *Exptl. Cell Research* **3,** 163 (1952).

further investigation. It has been shown that intact, dividing chick fibroblasts possess a region between the chromosomes at anaphase which absorbs ultraviolet at 265 mμ to a significantly higher extent than does the adjacent cytoplasm.[133]

2. Turnover Studies

Studies on the metabolism of the chromosomal nucleic acids in relation to mitosis have so far been concerned mainly with two questions: Is there a stable state of the nucleic acids in the resting nucleus or not, and, secondly, during which stage of the cell cycle are the nucleic acids synthesized for the next division?

The PNA-fraction of mammalian cells and nuclei has in most instances been found to be rapidly and extensively renewed.[134-137] Whether this also applies to the chromosomal PNA is unknown. The question of the biochemical stability of DNA will be discussed at length in Chapter 26, but a few of the salient features may be briefly summarized here.

The use of P^{32} has indicated that there is no significant turnover of the DNA-phosphorus in resting tissue representing a stable stage of the cell cycle[112,138,139] but the incorporation of P^{32} increases in proportion to the mitotic index of growing tissues.[138, 140,141] The same state of affairs has been found with N^{15}-labeled adenine,[142] and in some cases also with N^{15}-labeled glycine.[143] Once formed, the DNA molecule appears to be stable; using regenerating liver and labeled adenine Bendich[144] calculated a "half-life" of about 50 days for the DNA-fractions as compared with about 8 days for PNA. Using other precursors (formate, glycine, orotic acid, cytidine, and deoxyribosides), Hammarsten, Reichard, and others[145-149] have thrown a different light

[133] H. G. Davies, *Exptl. Cell Research* **3,** 453 (1952).
[134] D. Shemin and D. Rittenberg, *J. Biol. Chem.* **153,** 401 (1944).
[135] E. Hammarsten and G. Hevesy, *Acta Physiol. Scand.* **11,** 355 (1946).
[136] A. Bergstrand, N. A. Eliasson, E. Hammarsten, B. Norberg, P. Reichard, and H. von Ubisch, *Cold Spring Harbor Symposia Quant. Biol.* **13,** 22 (1948).
[137] J. N. Davidson, (ed.), *in* "Isotopes in Biochemistry," p. 175. Churchill, London, 1951.
[138] G. Hevesy, "Radioactive Indicators." Interscience, New York, 1948; *Advances in Biol. and Med. Phys.* **1:** 409 (1948).
[139] A. Marshak, *J. Cellular Comp. Physiol.* **32,** 381 (1948).
[140] E. Andreasen and J. Ottesen, *Acta Physiol. Scand.* **5,** 237 (1945).
[141] E. E. Osgood, H. Tivey, K. B. Davidson, A. J. Seaman, and J. G. Li, *Cancer* **5,** 331 (1952).
[142] R. Abrams and J. M. Goldinger, *Arch. Biochem. and Biophys.* **35,** 243 (1952).
[143] E. Hammarsten, B. Thorell, S. E. G. Åqvist, N. Eliasson, and L. Åkerman, *Exptl. Cell Research* **5,** 404 (1953).
[144] A. Bendich, *Exptl. Cell Research* Suppl. 2, 181 1952.
[145] E. Hammarsten, P. Reichard, and E. Saluste, *J. Biol. Chem.* **183,** 105 (1950).
[146] P. Reichard and B. Estborn, *J. Biol. Chem.* **188,** 839 (1951).
[147] D. Elwyn and D. B. Sprinson, *J. Am. Chem. Soc.* **72,** 3317 (1950).
[148] G. A. LePage and C. Heidelberger, *J. Biol. Chem.* **188,** 593 (1951).
[149] P. Reichard, *Acta Chem. Scand.* **3,** 422 (1949).

on the biochemical stability of DNA by showing that different precursors give different rates of incorporation into DNA. Until we know the synthetic pathways to DNA, the turnover studies hitherto made are difficult to interpret.

There are also indications of a heterogenous metabolism within different fractions of DNA. In regenerating liver, the incorporation of formate-C^{14} into the guanine of that fraction of DNA which is insoluble in cold physiological saline was 25% greater than into the guanine of a DNA-fraction remaining soluble. Adenine showed the reverse.[144] The field of metabolically, and probably also functionally, different DNA in the same set of chromosomes is just beginning to be explored, and many interesting findings can be expected in the near future.

As has been indicated, only the use of a proper precursor can be supposed to disclose the renewal of DNA in relation to the different stages of the cell cycle, but up to the present time P^{32} as phosphate has mainly been used for the autoradiographic detection of the uptake in single cells.[150,151] Root cells of *Vicia faba* were treated with P^{32}, and 4 hours later about 20 % of the nucleic of meristematic cells after extraction of lipid- and acid-soluble phosphorus showed autoradiographs, while cells in division showed none. It has been suggested by way of explanation that a cell which is preparing to divide synthesizes DNA during the first part of interphase. This explanation also gives a period of about 6 to 8 hours between the end of the synthesis and the beginning of visible prophase. During actual cell division little or no synthesis could be detected, and a cell which has completed its last division does not incorporate significant amounts of P^{32} in the nucleic acids.

The question of the stability and retention of DNA-phosphorus in resting cells has led to some interesting suggestions, some of which may be mentioned. For example, Hevesy,[138] and later Stevens, Daoust, and Leblond[152] have calculated from the quantitative relationship between the uptake of P^{32} in the DNA and the increments in mass or in the number of nuclei that about twice as much DNA-phosphorus is produced as can be accounted for by newly formed nuclei.[138,152,153] For example, in the liver it was calculated that 0.71% of the cells were newly formed per day and 1.2% new DNA-phosphorus, in intestinal mucosa 54% new cells and 95 to 114% new DNA-phosphorus. It was therefore suggested that the cell cycle is associated with a replacement of each of the mother DNA molecules by two new daughter molecules. The mechanism whereby the structural pattern might be kept intact under such circumstances is not understood, however, and until we know more about the mechanism of incorporation of P^{32} into the DNA molecule the question is open.

In general terms, it may be said that the extended chromosomes of the interphase or "resting" nucleus probably form the structural basis for the chains of syntheses, both of new chromosomal nucleic acids for the next

[150] A. Howard and S. R. Pelc, *Exptl. Cell Research* **2**, 178 (1951).

[151] A. Howard and S. R. Pelc, *in* "Isotopes in Biochemistry," (J. N. Davidson, ed.), Churchill, London, 1951.

[152] C. E. Stevens, R. Daoust, and C. P. Leblond, *J. Biol. Chem.* **202**, 177 (1953).

[153] R. Daoust, F. D. Bertalanffy, and C. P. Leblond, *J. Biol. Chem.* **207**, 405 (1954).

division and of specific substances of enzymic character for the function of the tissue elements.

IV. Concluding Remarks

In the preceding sections an attempt has been made to give a balanced picture of what in the author's opinion, are the conspicuous facts about the chromosomal nucleic acids during the cell cycle. The physiological importance of these compounds is, however, still unknown. Among the chromosomal substances there are representatives of the hereditary material. DNA has been regarded as the most characteristic chromosomal constituent and the view, which can be traced back to the work of Miescher, that DNA represents the genes received strong support when the bacterial transforming factors were discovered[154] (see Chapter 27). The apparent chemical homogeneity of DNA preparations from different sources, however, was for long difficult to reconcile with the manifold genic characters. The work of Chargaff and others[155-157] surmounted this obstacle (Chapter 10), and the recent results of X-ray diffraction work by Watson and Crick and Wilkins *et al.*[158-160] (Chapter 13) leave very little to be desired as regards possibilities of structural variations in the DNA molecule.

The problem of how the chromosomal nucleic acids exert their effects on the cell and the organism may be presented in two questions: Are they connected with protein synthesis, and, in particular, with the formation of specific intracellular enzymes? Is their main task structural and/or catalytic? One obvious difficulty in getting an answer is the nature of the substances themselves. The nucleic acids and the nucleoproteins prepared according to the present available procedures, unlike, for instance, many of the fairly well-known enzymic energy systems, are no doubt very different from the compounds as they occur in the cell. The adequate study of these questions, therefore, needs much further work of a fundamental biochemical and biophysical character.

[154] O. T. Avery, C. M. MacLeod, and M. McCarty, *J. Exptl. Med.*, **79**, 137 (1944).
[155] E. Chargaff, *Federation Proc.* **10**, 654 (1951).
[156] E. Chargaff, C. F. Crampton, and Rakowa Lipshitz, *Nature* **172**, 289 (1953).
[157] G. L. Brown and M. Watson, *Nature* **172**, 339 (1953).
[158] J. D. Watson and F. H. C. Crick, *Nature* **171**, 737 (1953).
[159] M. H. F. Wilkins, A. R. Stokes, and H. R. Wilson, *Nature* **171**, 740 (1953).
[160] R. E. Franklin and R. G. Gosling, *Nature* **171**, 745 (1953).

CHAPTER 21

The Cytoplasm

GEORGE H. HOGEBOOM and WALTER C. SCHNEIDER

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I. Introduction

1. General

Although for many years cytologists have been aware of the great complexity of the structural organization of the cell cytoplasm, concrete in-

formation relating to structural detail has until recently been severely curtailed by the inherent limitations in the resolving power of the light microscope. It is now apparent, however, that electron microscopy is capable of surmounting this impasse to such a degree that structures of molecular dimensions can, at least in theory, be characterized morphologically. The result is that cytology is no longer a static science and that, among other advances in the field, a much more complete picture of the cell cytoplasm is being rapidly unfolded.

Concurrently, a relatively new and still somewhat controversial area of research, generally referred to as cytochemistry, has produced a considerable mass of data relating the biochemical organization of the cell to its structural organization. Like the new cytology, however, cytochemistry is suffering from growing pains in that it is at present strictly in a methodological stage and its methods are as yet imperfect. In the present chapter, an attempt is made to discuss the uses and limitations of the cytochemical technics now available and to summarize the results that appear to be based on sound experimentation. The reader is referred to a number of other recent reviews,[1-13] either general in nature or dealing with specific aspects of cytochemistry, and presenting, in some instances, points of view different from that of the present authors.

2. Cytology of the Cytoplasm

By far the greatest proportion of data pertaining to the biochemical properties of cytoplasmic components has been obtained in studies of mammalian liver. This tissue is convenient for cytochemical investigations, particularly for cell fractionation experiments, because in terms of total mass (see below) it is composed largely of what appears to be a single type of cell containing abundant cytoplasm and readily disrupted by mechanical means without undue damage to the intracellular elements.

[1] A. L. Dounce, *in* "The Enzymes" (Sumner and Myrbäck, eds.), Vol. 1, p. 188. Academic Press, New York, 1950.
[2] A. L. Dounce, *J. Cellular Comp. Physiol.* **39,** Suppl. 2, 43 (1952).
[3] C. de Duve, *Exposés ann. biochim. méd.* **14,** 47 (1952).
[4] G. H. Hogeboom, *Federation Proc.* **10,** 640 (1951).
[5] G. H. Hogeboom, W. C. Schneider, and M. J. Striebich, *Cancer Research,* **13,** 617 (1953).
[6] H. Holter, *Advances in Enzymol.* **13,** 1 (1952).
[7] K. Lang, *Colloq. deut. Ges. physiol. Chem. Mosbach-Baden,* p. 24 (1951).
[8] W. C. Schneider and G. H. Hogeboom, *Cancer Research* **11,** 1 (1951).
[9] W. C. Schneider, *J. Histochem. and Cytochem.* **1,** 212 (1953).
[10] J. F. Danielli, "Cytochemistry, a Critical Approach." Wiley, New York, 1953.
[11] J. R. G. Bradfield, *Biol. Revs.* **25,** 113 (1950).
[12] H. Holter and K. Linderstrøm-Lang, *Physiol. Revs.* **31,** 432 (1951).
[13] D. E. Green, *J. Cellular Comp. Physiol.* **39,** Suppl. 2, 75 (1952).

The mitochondria are the most prominent cytoplasmic structures of the liver cell that can be seen in the light microscope. Simultaneous counts of free nuclei and mitochondria in liver homogenates have indicated that the number of mitochondria per cell is of the order of 400.[14] These ubiquitous cell orgenelles are characterized generally by a filamentous, rod-like, or granular form and by certain well-defined staining properties.[15,16] Cytological observations have, in fact, led to the belief that the mitochondria of various cells and even of the cells of various species are structurally and functionally similar—a view that has received strong support from recent electron microscopic studies of the internal structure of mitochondria.[17,18] Thus, by the use of extremely thin tissue sections, Palade[17] has shown that the mitochondria of a wide variety of cells uniformly possess a surface membrane 7 to 8 mμ in thickness, a system of parallel regularly spaced ridges protruding from the inside surface of the membrane towards the interior, and an internal matrix containing occasional minute granules, but otherwise structureless. Similar findings have been made independently by Sjöstrand and Rhodin.[18] Other structures of microscopically visible dimensions within the liver cell cytoplasm are small spherical granules, 0.5 to 1 μ in diameter, located near the periphery of the cell, and larger lipid droplets, 2 to 3 μ in diameter, located in the interior. Both of these components are demonstrable in fresh preparations by their affinity for neutral red;[19] the former are considered to be secretory granules, presumably containing bile. The unsettled status of the Golgi apparatus in liver will be discussed later. In the cells of tissues other than liver, a number of additional, specialized cytoplasmic structures can, of course, be seen. These include pigment granules, other types of secretory granules, inclusion bodies, and the various structures related to the myofibrils of muscle cells.

One of the main contributions of electron microscopy to cytology has been the disclosure of the structural details of the optically empty ground substance or hyaloplasm of the cell. Thus, in all cells examined, a rather large proportion of the cytoplasmic volume has been found to contain a complicated reticular network that is too small to be seen in the light

[14] G. H. Hogeboom, W. C. Schneider, and M. J. Striebich, *J. Biol. Chem.* **196,** 111 (1952); cf. C. Allard, R. Mathieu, G. de Lamirande, and A. Cantero, *Cancer Research* **12,** 407 (1952).

[15] E. V. Cowdry, *in* "General Cytology" (Cowdry, ed.), p. 113. Univ. of Chicago Press, Chicago, 1924.

[16] G. H. Bourne, *in* "Cytology and Cell Physiology" (Bourne, ed.), 2nd ed., p. 313. Clarendon Press, Oxford, 1951.

[17] G. E. Palade, *Anat. Record* **114,** 427 (1952); *J. Histochem. and Cytochem.* **1,** 188 (1953).

[18] F. S. Sjöstrand and J. Rhodin, *Nature* **171,** 30 (1953).

[19] G. H. Hogeboom, W. C. Schneider, and G. E. Palade, *J. Biol. Chem.* **172,** 619 (1948).

microscope.[20-23] This structure, which is referred to as the endoplasmic reticulum[24] or the ergastoplasm,[22] probably corresponds to the basophilic substance seen in fixed and stained cells under the light microscope. Its detailed morphology is still somewhat controversial; some investigators[21] consider it to be in the form of vesicles and canaliculi, whereas others[25] believe it to be lamellar in form.

II. Cytochemical Methods

1. General

Three methods are available for the study of the chemical composition and the biological function of intracellular structures: (1) histochemical methods, (2) submicromethods capable of direct chemical and enzymic determinations on single cells or portions thereof, and (3) the cell fractionation technic. All of these procedures suffer from certain inherent defects that prevent any one of them from providing definitive answers to all cytochemical problems. It will become evident, however, that the third method has provided a much larger amount of information than has either of the other two. Although the results considered in this chapter will therefore be limited mainly to those of the cell fractionation technic, it is important to consider the advantages and limitations of the other cytochemical methods as well.

2. Histochemical Methods

In the histochemical localization of a chemical compound or of an enzyme, it is necessary to visualize the compound or the product of the enzymic reaction in a tissue section. Physical methods have been developed permitting the quantitative determination of the nucleic acids (by ultraviolet microspectrophotometry[26]) and of certain elements (by X-ray spectrometry[27]) at subcellular levels. These technics, although in some instances capable of providing information that can be obtained in no other way, have certain limitations. The former (see Chapter 17) suffers from the disadvantage that it is unable to distinguish between the two types of nucleic acids or between nucleic acids and other related compounds normally found in cells in high concentrations (e.g., the adenosine tri-, di-, and

[20] K. R. Porter and H. P. Thompson, *Cancer Research* **7**, 431 (1947).
[21] A. J. Dalton, H. Kahler, M. J. Striebich, and B. J. Lloyd, *J. Natl. Cancer Inst.* **11**, 439 (1950).
[22] W. Bernhard, A. Gautier, and C. Oberling, *Compt. rend. soc. biol.* **145**, 566 (1951).
[23] G. E. Palade and K. R. Porter, *Anat. Record* **112**, 68 (1952).
[24] K. R. Porter, *J. Exptl. Med.* **97**, 727 (1953).
[25] A. J. Dalton, *Intern. Rev. Cytol.* **2**, 403 (1953).
[26] T. Caspersson, "Cell Growth and Cell Function." Norton, New York, 1950.
[27] A. Engström, *Acta Radiol.* Suppl. 63 (1946).

monophosphates, certain coenzymes, and ascorbic acid). The X-ray absorption method is not as yet capable of scanning areas smaller than 10 μ x 10 μ and consequently cannot be applied to cell structures smaller than the nucleus. A number of other histochemical technics depend on the formation of visible products, usually colored, as the result of a chemical reaction or physical combination taking place within the cell. Staining methods have, for example, been used as a means of the qualitative differentiation in tissue sections between the two types of nucleic acid. Here the mechanism of the reaction is not well understood, and previously accepted claims as to the specificity attained have received a rather serious setback by the recent observations of Alfert,[28] who found that methyl green, which has generally been considered a specific stain for DNA, would also stain PNA in certain tissues and, furthermore, would stain PNA in refractory tissues if the fixative contained high concentrations of formaldehyde. The Feulgen reaction[29] is a good example of a histochemical method involving a chemical reaction and is also generally considered specific for DNA. It is discussed in detail in Chapter 17. As in the case of most histochemical tests involving chemical reactions, the Feulgen reaction is, in fact, specific only for a certain chemical grouping (the aldehyde group). Although the manner in which the reaction is carried out apparently contributes to its specificity for DNA, there is some question as to whether the reaction product and the DNA are localized at the same intracellular site.[10] The latter problem represents a source of error that is often shared by histochemical procedures providing the localization of enzymic reactions.[30] Here a further complication is introduced by the fact that enzymes are generally labile molecules and are therefore often inactivated to a considerable extent by the necessary procedures of fixation and embedding. As a result, the possiblity must be considered that the enzyme can be selectively destroyed in certain cells or even in different parts of the same cell.

Another important consideration relating to histochemical methods arises from the question as to whether they can demonstrate the localization of enzymes and other compounds at subcellular levels. Danielli[31] apparently feels that the Gomori technics for the detection of alkaline and acid phosphatases,[30] for example, are capable, under properly controlled conditions, of localizing the enzymes within cells. Novikoff[32,33] and Palade,[34] on the other hand, have made a careful comparison of the results of these

[28] M. Alfert, *Biol. Bull.* **103,** 145 (1952).
[29] R. Feulgen and H. Rossenbeck, *Z. physiol. Chem.* **135,** 203 (1924).
[30] G. Gomori, "Microscopic Histochemistry." Univ. of Chicago Press, Chicago, 1952.
[31] J. F. Danielli, *J. Exptl. Biol.* **22,** 110 (1946).
[32] A. B. Novikoff, *Science* **113,** 320 (1951).
[33] A. B. Novikoff, *Exptl. Cell Research* Suppl. 2, 123 (1952).
[34] G. E. Palade, *J. Exptl. Med.* **94,** 535 (1951).

histochemical methods with direct enzyme assays of cell fractions isolated by centrifugation and find that entirely different results are obtained by the two procedures. The histochemical tests indicated that phosphatase activity is confined mainly to the nucleus (see Chapter 18), whereas the cell fractionation experiments demonstrated apparent localization in the cytoplasm. A convincing explanation for this discrepancy was offered by the latter investigators in the finding that cell nuclei have a strong affinity for calcium and lead phosphates. Since these two metallic ions are used to precipitate the inorganic phosphate liberated by alkaline and acid phosphatase, it is apparent that the histochemical methods are capable of producing an artifact and are therefore probably not suitable for demonstrating the intracellular distribution of the enzymes. It should be pointed out that these data do not necessarily invalidate conclusions derived from histochemical studies of the distribution of phosphatases among histologically defined areas of various tissues.[31]

Recent reports have indicated, however, that certain other histochemical methods may provide accurate intracellular localization of enzymes. Thus, Hoffmann *et al.*[35] found that cytochrome oxidase, as demonstrated by the Nadi reaction, is localized exclusively in the mitochondria of lymphoid and myeloid cells. Mitochondrial localization of succinic dehydrogenase in cells was also demonstrated by Malaty and Bourne,[36] who used tetrazolium salts to give a colored reaction product. These findings have confirmed results obtained by the cell fractionation technic (see below). It would therefore appear that histochemical methods can provide reliable information in some instances but that the data should be checked by independent means. It is, in addition, reassuring to note that, by the application of both histochemical and cell fractionation methods, it has been possible to obtain confirmatory results on the one hand and to detect artifacts on the other. Although the possibility must be entertained that the results of both methods may be false, their simultaneous use offers considerable promise as a means of solving cytochemical problems.

3. Submicromethods

The submicromethods[6,12,37] are theoretically capable of the quantitative analysis of single cells and their structural components and in this respect represent the ideal cytochemical tool. In practice, however, the results do not indicate that this ideal has been achieved except in special instances, because it has not as yet been possible to increase the sensitivity of enzymic assays and chemical analyses sufficiently to permit the study of any but

[35] G. T. Hoffman, A. Rottino, and K. G. Stern, *Blood* **6,** 1051 (1951).
[36] H. A. Malaty and G. H. Bourne, *Nature* **171,** 295 (1953).
[37] K. Linderstrøm-Lang, *Harvey Lectures* **34,** 214 (1938–39).

the largest unicellular organisms. Definitive cytochemical data have been obtained, however, from the fragments of these cells, the conclusions being dependent upon correlations between the amount of enzymic activity and the presence of a given cell structure in an isolated fragment.[38] According to recent reviews,[6,12] the accomplishments in this field are limited to correlations demonstrating the localization of amylase,[38] proteinase,[39] and succinic dehydrogenase[40] in the mitochondria of amoebas. Although it is obvious that the results obtained with the submicrotechnics are not very extensive, the data have been invaluable in that they have provided independent confirmation of certain findings obtained with other cytochemical methods, notably that of cell fractionation.

4. The Cell Fractionation Technic

a. Advantages and Limitations

In the technic of cell fractionation, the cells of a tissue are mechanically disrupted, and the nuclei, mitochondria, and other cellular components are released into a suitable medium from which they can be isolated by differential centrifugation. This method has the advantage of permitting the isolation of all the particulate components of cells from a single sample of tissue in a yield and degree of purity sufficient to permit an accurate comparison of the properties of the isolated structures. Furthermore, the fact that the cell fractions can be obtained in almost unlimited quantities makes it possible to utilize accepted analytical methods in studies of the biochemical properties of the isolated cell components and thus to keep pace with the rapid advances in biochemistry. It is obvious, therefore, that the cell fractionation procedure is capable of yielding more information about the properties of cell structures than is any of the other cytochemical methods available. For this reason, it is extremely important to define the limitations of the technic. It is necessary first of all to recognize that the tissues commonly used for the isolation of cell structures do not represent uniform populations of a single cell type. Thus, although mammalian liver consists predominantly of hepatic parenchymal cells (85 to 80% by *volume*), 40 to 50% of the total *number* of cells are nonparenchymal in type.[41] Since it is therefore apparent that the nonparenchymal cells

[38] H. Holter and W. L. Doyle, *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **22,** 219 (1938).

[39] H. Holter and S. Løvtrup, *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **27,** 27 (1949).

[40] N. Andresen, F. Engel, and H. Holter, *Compt. rend. lab. Carlsberg, Sér. chim.* **27,** 408 (1951).

[41] M. E. Wilson, R. E. Stowell, H. O. Yokoyama, and K. K. Tsuboi, *Cancer Research* **13,** 86 (1953).

account for a relatively small proportion of the total cytoplasm of liver, the finding that they comprise 40 to 50% of the total cell population is of significance only in investigations in which the composition of the average parenchymal cell is calculated on the basis of the total number of nuclei present.[42,43] Another possible source of error involved in extrapolating measurements made on large numbers of liver cells to that of the single average liver cell lies in the fact that certain histochemical tests have suggested that liver cells appearing to be morphologically identical may have considerably different chemical compositions and enzymic properties.[10] If these observations can be accepted as correct, they would represent an important limitation of the cell fractionation technic and an excellent example of the way in which histochemical and cell fractionation technics can supplement each other.

Finally, it is necessary to consider an argument frequently used in peremptorily dismissing cell fractionation as a cytochemical tool,[10,11,44-46] namely, that at the moment of or immediately after cell rupture, so many artifacts occur (such as morphological alterations, adsorption, redistribution autolysis, etc.) that it is utterly useless to isolate the cell structures and study their properties. Since this argument is usually set forth without supporting experimental evidence and without the submission of an adequate alternative, it obviously represents neither a realistic nor a constructive point of view. As noted on several occasions,[4,5,8] while it is important to recognize that such artifacts can occur, it is at the same time possible to carry out experiments to determine whether they *do* occur. The latter approach has been amply justified. Thus in several investigations to be described later, strong evidence has been obtained for the absence of absorption and redistribution artifacts during cell fractionation. Furthermore, certain results obtained with the cell fractionation procedure have been confirmed by entirely independent methods, e.g., by histochemical experiments[35,36] and submicromeasurements.[40]

b. Requirements Defining an Adequate Isolation Procedure.

In employing the cell fractionation technic for the studies of the intracellular distribution of enzymes and other compounds, it must first be borne in mind that the procedures presently available for the isolation of cell components are by no means perfect. Furthermore, as already indicated, in the absence of other productive methods of approach to cytochemical problems, the data obtained by the cell fractionation

[42] M. J. Striebich, E. Shelton, and W. C. Schneider, *Cancer Research* **13,** 279 (1953).
[43] W. C. Schneider, G. H. Hogeboom, E. Shelton, and M. J. Striebich, *Cancer Research* **13,** 285 (1953).
[44] J. F. Danielli, *Nature* **157,** 755 (1946).
[45] R. Chambers, *Cancer Research* **10,** 210 (1950).
[46] J. F. Danielli, *Nature* **157,** 755 (1946).

technic cannot often be checked by independent means. Until this unfortunate situation is resolved, therefore, it is imperative both to set up certain requirements defining an adequate isolation procedure and to appraise critically the results obtained in terms of the possible defects of the methods used.

Obviously, it is much simpler to define than to devise an adequate method for the isolation of a cell structure. It should provide the structure in unaltered form, in homogeneous preparations, and in good yield. The term, unaltered, denotes the absence of both morphological and biochemical changes. Thus it is difficult to believe that a cytologically altered structure could possibly be biochemically intact. Since cytological methods are practically the only means by which structures within living cells have been characterized, however, these same methods must be relied upon in an assessment of the integrity of cell structures isolated from their cellular environment. The use of cytological criteria of integrity has been criticized on the ground that isolated cell structures that are morphologically intact may not be biochemically intact.[47] Mitochondria, for example, isolated in morphologically and cytologically *unaltered* form, have been found less active in catalyzing certain enzymic reactions than mitochondria isolated in an altered form (cf. footnote 8). This does not constitute justification for disregarding the cytological observations and for substituting biochemical criteria of integrity based upon enzymic activity, because it is quite obvious that we are not as yet able to arrive at a definition of biochemical integrity. Nevertheless, it must be admitted that the adequacy of cytological criteria is often questionable, and it is therefore hoped that an answer to the difficult problem of detecting physical and chemical alterations in isolated cell structures will result from a continuous search for better methods and from the objective, critical evaluation of extensive data based upon a diligent alertness for possible artifacts.

The necessity for striving for homogeneity in the isolation of cell structures is obvious. We must go even farther than this, however, for the term, homogeneity, can only be used in a relative sense, and quantitative methods of analysis must therefore be devised for determining the degree of homogeneity attained. This is an axiom in the field of chemistry. In cytochemistry and in the absence of a better method, microscopic examination has usually been employed in the past as the chief means of estimating the purity of preparations of isolated cell structures. Unfortunately, cursory microscopic examination is not in itself an objective, quantitative method and can therefore only be used as a means of ruling out gross contamination. For this reason, the assignment of a biochemical property to a cell structure can be safely made only when that property is present in the isolated preparation in a relatively high concentration, for example, in a concentration exceeding that in the whole tissue.[4,5,8,48] This principle is obviously arbitrary and will certainly have to be abandoned when improved methods are available.

It should be mentioned, however, that several quantitative methods are now available that in certain instances permit an estimate of the extent of cross-contamination between cell fractions. The amount of nuclear material in a preparation can, for example, be determined by the diphenylamine or other colorimetric reaction for DNA. Furthermore, the number of free mitochondria in fractions isolated in sucrose solutions can be estimated by a direct counting procedure consisting essentially of making a suitable dilution in hypotonic (0.125 *M*) sucrose and identifying and counting the swollen mitochondria in a bacterial counting chamber under the phase micro-

[47] A. L. Dounce, *Cancer Research* **11**, 562 (1951).

[48] G. H. Hogeboom, and W. C. Schneider, *J. Biol. Chem.* **186**, 417 (1950).

scope.[14,49] The number of unbroken liver cells can also be determined by direct count. The relatively high PNA content of liver microsomes makes it possible to determine the degree of separation of these particles from mitochondria, which have a low PNA content.

The question of yield is also of importance. To isolate 5% of the mitochondria or microsomes of a tissue and assume that analysis of these preparations are representative of the whole tissue is not a sound procedure. This is particularly true in the case of microsomes, which are a heterogeneous group of particles with respect to both size and function and, therefore, might readily be fractionated in a procedure leading to low yields. It is also possible that the components of different cell types within a single tissue vary sufficiently in their properties to lead to similar difficulties when poor yields are obtained.

Another important point arises from the fact that a high yield makes it possible to determine the proportion of the enzyme activity of the whole tissue recovered in each cell fraction. Recovery values can be very enlightening. Many enzymes that are said to reside in the liver cell nucleus, for example, have been found in isolated preparations in a concentration of 40 to 100% of that in the whole tissue.[1,2] When it is realized that the nucleus of the liver cell accounts for about 10% of the total mass of the cell, it is apparent that these concentration values of 40 to 100% would have represented enzyme recoveries of only 4 to 10% *if* the yield of nuclei had been complete, and in these experiments the yield of nuclei was far from complete. It is apparent that cell fractionation procedures are fraught with too many uncertainties to permit definitive conclusions in the face of enzyme recoveries of this order.

Attention to yield and recovery also makes it possible to draw up a balance sheet, a point that for some reason has had to be defended on numerous occasions[4,5,8,48] (cf. footnotes 32, 50). It is not enough to determine the enzyme activity of a single fraction with the object of interpreting that analysis in terms of the whole tissue. The original whole tissue and all fractions must be analyzed. If the sum of the activity of the fractions equals within reasonable limits the activity of the whole tissue, then the method of enzyme assay is likely to be adequate, at least for cytochemical purposes. If not, something is wrong with the enzyme determination. The necessity for drawing up balance sheets becomes obvious when one considers the inherent difficulties involved in enzyme assays, especially in dealing with complicated mixtures of unknown biochemical composition. Enzyme assays are based on determinations of reaction rates. Reaction rates can be markedly affected by a number of factors, including inhibitors, activators, interference or competition as a result of side reactions, relatively slight changes in the pH or ionic strength of reaction mixtures, and permeability barriers set up by membranes. Unfortunately, one of the most common sources of misleading data, particularly in certain investigations of complex enzyme systems, has been the probability that the enzyme under study was not the rate-limiting component of the reaction. This situation has arisen a number of times from measurements of oxygen uptake when various oxidizable substrates are added either to isolated mitochondria or to heterogeneous particulate preparations containing mitochondria, e.g., "cyclophorase."[13] When it is remembered, as will be demonstrated later, that cytochrome oxidase is exclusively localized in the mitochondrion, it becomes obvious that *this is the only cell structure capable of taking up oxygen in any reaction leading to the reduction of cytochrome c*. Oxygen uptake will therefore occur even if the enzyme supposedly under study (e.g., a dehydrogenase)

[49] E. Shelton, W. C. Schneider, and M. J. Striebich, *Exptl. Cell Research* **4**, 32 (1953).
[50] H. Stern, V. G. Allfrey, A. E. Mirsky, and H. Saetren, *J. Gen. Physiol.* **35**, 559 (1952).

TABLE I

INTRACELLULAR DISTRIBUTION OF ISOCITRIC DEHYDROGENASE[48]

Preparation	Per cent of original activity	
	Isocitric dehydrogenase[a]	Oxidation of D-isocitrate[b]
Homogenate	100	100
Nuclear fraction	3	7
Mitochondria	12	23
Microsomes	0.9	1
Supernatant	82	1 *Ca.*

[a] Activity determined by following spectrophotometrically the rate of reduction of TPN on addition of D-isocitrate.

[b] Activity determined by measuring the rate of oxygen uptake on addition of D-isocitrate in the presence of TPN, cytochrome c, and ATP.

is present in the mitochondrial fraction in only trace amounts. An excellent example of this phenomenon was reported in a study of the intracellular distribution of isocitric dehydrogenase.[48] As is shown in Table I, when the dehydrogenase was determined directly by following the rate of reduction of TPN on addition of D-isocitrate, the recovery of enzyme activity was essentially complete, 82% being in the supernatant or soluble fraction and only 12% in the mitochondrial fraction. When D-isocitrate was added as an oxidizable substrate in the presence of TPN and cytochrome c, however, only the mitochondrial and nuclear fractions (the latter because of its mitochondrial content) took up significant amounts of oxygen, and a very low recovery was obtained. It can be seen that the oxygen uptake determinations gave a completely false picture of the distribution of isocitric dehydrogenase and that the inadequacy of the enzyme assay was indicated by a low recovery. The results of further experiments indicated, in fact, that TPN-cytochrome c reductase, rather than isocitric dehydrogenase, was the rate-limiting enzyme in the oxidation of isocitrate. The value of drawing up balance sheets and the necessity for *direct* determinations of enzyme activities are thus clearly demonstrated.

c. Method of Cell Disruption

The general plan followed in the isolation of cellular components is to disrupt the cells in a suitable medium and to segregate the liberated cell structures by means of differential centrifugation. The method used for the disruption of cells is of primary importance, since it must stop short of damage to the nucleus, mitochondria, and other cell structures, while producing almost quantitative breakage of cells. The Potter-Elvehjem homogenizer,[51] which has been mentioned in Chapter 18, has satisfactorily met these requirements. It has been found in the writers' laboratory, for example, that a homogenizer of this type, consisting of a pestle machined from the plastic Kel-F (a monochlorotrifluoroethylene polymer) to fit a smooth-walled pyrex test tube and rotated at 600 to 1000 r.p.m., is ideally suited for the disruption of the cells of soft tissues (e.g., liver, kidney, and certain tumors). Furthermore, a higher percentage of cells are disrupted if there is a slight clearance rather than a tight fit

[51] V. R. Potter and C. A. Elvehjem, *J. Biol. Chem.* **114**, 495 (1936).

between the pestle and test tube. Apparently, the major factor in cell disruption with this homogenizer is the rapidity with which the tissue suspension can be forced between the wall of the tube and the rotating pestle rather than the amount of clearance that exists between the pestle and the tube wall. In the case of liver, homogenization for 2 minutes is sufficient to break over 95% of the cells.[14] Other investigations have indicated that longer periods of homogenization might lead to disruption of cellular particulates as well.[52] Since all-glass homogenizers were used in the latter experiments, it is possible, however, that fragments of glass may have contributed to the disruption of the particles. The use of homogenizers is discussed also in Chapter 18.

d. The Effect of Various Media on Cytoplasmic Particles and the Cytological Identification of the Components of Cell Fractions

The medium in which the cells are disrupted is of great importance in that it influences not only the morphological and cytological properties of cell structures, but also their physical and biochemical properties. In 1947, a reinvestigation of Claude's original method[53,54] for the fractionation of cytoplasmic particulates was undertaken by the present authors[19,55] with the aim of improving the yields and of identifying more positively the cellular elements present in the fractions. The experiments were mainly concerned with the "large-granule" fraction, which was thought by Claude to consist of a mixture of mitochondria and secretory granules.

When released into isotonic solutions, the large granules appeared as refractive spherical bodies, 0.5 to 2 μ in diameter. When exposed to hypotonic salt solutions or to water, they became greatly swollen, and as Claude had shown previously,[53,54] lost appreciable amounts of soluble material. It was also noted that marked aggregation of the granules occurred in the solutions of neutral electrolytes that had been employed previously as media, e.g., isotonic NaCl, KCl, and phosphate buffer.[8] The clumps of granules were of such a size that they sedimented together with nuclei and intact liver cells. It was therefore impossible to remove nuclei, intact liver cells, and connective tissue from the broken cell suspensions by centrifugation without sedimenting 40 to 80% of the large granules. When released into isotonic (0.25 *M*) sucrose, however, the granules showed no tendency to aggregate.[19] By means of a method described below, it was possible with this medium to separate efficiently the nuclei and microsomes from large granules and to obtain the latter in yields of 80% or greater.

The problem of identifying the structures present in the isolated large-granule fraction was simplified to a considerable degree by the finding that most of the granules were rod-like in shape when released into hypertonic solutions of sucrose.[19] When a sucrose concentration of 0.88 *M* was employed, the granules retained their morphological characteristics after having been separated from other cell constituents and washed several times in the centrifuge. It was evident that they were similar in size and shape to the mitochondria of the liver cell. In addition, they could be stained supravitally with Janus Green B, and, after fixation with osmium tetroxide, they showed the other staining characteristics that for many years had been used to

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[52] T. A. F. Quinlan-Watson and D. W. Dewey, *Australian J. Sci. Research* **B1,** 139 (1948).

[53] A. Claude, *J. Exptl. Med.* **84,** 51 (1946).

[54] A. Claude, *J. Exptl. Med.* **84,** 61 (1946).

[55] G. H. Hogeboom, W. C. Schneider, and G. E. Palade, *Proc. Soc. Exptl. Biol. Med.* **65,** 320 (1947).

identify mitochondria. The isolated and washed preparations contained no elements stainable with neutral red (e.g., secretory granules and lipid droplets) and, as far as could be determined by examination in the electron microscope, contained very little material of submicroscopic dimensions. It was therefore concluded that the preparations were essentially homogeneous suspensions of mitochondria.

A number of subsequent observations have indicated that the "large-granule" fraction isolated from liver homogenates prepared in isotonic (0.25 *M*) sucrose solution also consists of mitochondria, despite the fact that in this medium the particles are uniformly spherical and are not readily stained by Janus Green B. Thus, after appropriate fixation, the granules are intensely colored by mitochondrial stains. Furthermore, when the suspension is diluted somewhat with water and examined in the phase microscope, the swollen granules show the typical morphological characteristics of mitochondria described by Zollinger[56] and Harmon,[57] i.e., a delicate, transparent sphere capped at one or both poles by crescent-shaped, dense material. A number of biochemical studies[48,58] have indicated that there are no appreciable differences in the properties of liver mitochondria isolated in isotonic (0.25 *M*) or hypertonic (0.88 *M*) sucrose solution. The latter medium, because of its density and viscosity, requires relatively high centrifugal forces in the fractionation of liver homogenates and on occasion makes it necessary to add such large amounts of sucrose to reaction mixtures that the activity of some enzymes is inhibited (cf. footnote 8). For these reasons, the medium of choice for the isolation of mitochondria would appear at present to be isotonic sucrose. It has been the authors' experience that the addition of salts to the medium, even at low concentrations, causes sufficient aggregation of both mitochondria and microsomes to interfere considerably with the subsequent fractionation of liver homogenates.

The components of the microsomal fraction have not is yet been identified with certainty, mainly because it is only since the advent of electron microscopy that the fine details of cytoplasmic structure have been disclosed. Recent observations of Porter[24] have indicated, however, that isolated microsomes include the endoplasmic reticulum of the cytoplasm. Since this structural network is probably identical to the basophilic ground substance of cells,[22] Claude's[54] initial suggestion that the microsomes actually represent the basophilic ground substance seems well founded. Additional confirmation of this view is found in the work of Brenner.[59] From time to time, however, microsomes have been considered to be artifacts derived from other cell structures, e.g., from mitochondria.[13,57] That the latter concept is of doubtful validity will be demonstrated later in a discussion of the biochemical properties of the microsomal fraction.

Finally, it should be pointed out that the conditions for the fractionation of liver may not be applicable to other tissue. It may be necessary to use a different means of homogenization, a different isolation medium, or a modified isolation procedure to attain the desired separations. In using cell fractionation methods, it is essential that careful microscopic examination of the fractions be made and supplemented with suitable cytological methods for the identification of the isolated cell structures. Unless such observations are made, the nature of the fractions isolated must be considered as highly questionable.

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[56] H. U. Zollinger, *Experientia* **6,** 14 (1950).

[57] J. W. Harmon, *Exptl. Cell Research* **1,** 394 (1950).

[58] W. C. Schneider and G. H. Hogeboom, *J. Biol. Chem.* **183,** 123 (1950).

[59] S. Brenner, *S. African J. Med. Sci.* **12,** 53 (1947).

e. Isolation of Mitochondria and Microsomes from Liver

(1) *Preliminary Steps.* It is often desirable to perfuse the livers in order to eliminate erythrocytes and other blood elements from the cell fractions. Reasonably complete perfusion can be obtained through the hepatic-portal vein in rats and the inferior vena cava in mice. A needle of appropriate size (22 to 25 gauge), fitted to a three-way stopcock, is inserted into the vein and held in place by a hemostat. The vena cava in the rat or the portal vein in the mouse is severed, and the liver is perfused first with cold isotonic saline until free of blood and subsequently with cold 0.25 or 0.88 *M* sucrose to remove the saline. Most of the connective tissue can then be eliminated and the organ reduced to a pulp when it is forced, by means of a tissue press, through a stainless steel or plastic disk, perforated by numerous 1-mm. holes. Recent experiments[60] have suggested, however, that the latter procedure results in damage to an appreciable number of nuclei and is therefore of questionable value. The whole liver or liver pulp is weighed and homogenized in 9 volumes of 0.25 or 0.88 *M* sucrose. In the authors' experience, the use of more concentrated tissue suspensions has reduced considerably the yield of mitochondria, and it has thus been desirable to prepare "10%" homogenates even when relatively large amounts of liver are fractionated.

The complete fractionation of liver homogenates prepared in 0.25 *M* sucrose will now be described. If 0.88 *M* sucrose is used as the medium, the fractionation involves similar steps, but higher centrifugal forces are required.[58] The entire procedure is carried out at a temperature of 5° or less.

(2) *Removal of Nuclei and Intact Liver Cells.* Ten milliliters of the homogenate is pipetted into each of two 30-ml. graduated test tubes and centrifuged at 2000 r.p.m. (700 *g*) in the International refrigerated centrifuge (horizontal head No. 269). An alternative procedure permitting somewhat more efficient separation of nuclei from mitochondria consists of layering 10 ml. of the homogenate over 10 ml. of 0.34 *M* sucrose and centrifuging at the same speed.[60,61] The supernatant is withdrawn with a capillary pipet, and the residue is resuspended by adding 4.0 ml. of 0.25 *M* sucrose to each tube and homogenizing for 10 to 15 seconds with a loosely fitting Kel-F pestle. The suspension is recentrifuged for 10 minutes at 2600 r.p.m. in the same head. The supernatant is removed and combined with the first supernatant to form the cytoplasmic extract. The residue or "nuclear fraction" is made up to a known volume by adding 0.25 *M* sucrose and rehomogenizing. It contains all the nuclei present in the homogenate, the residual intact liver cells, erythrocytes, and, as determined by actual count, about 10% of the original number of free mitochondria.[61] Although obviously inhomogeneous, this fraction is nevertheless useful in studies of the biochemical properties of the nucleus, since a quantitative recovery of nuclei is obtained and the contribution of the other cellular components present can be estimated from additional measurements. The usefulness of the fraction is still further increased if the homogenate is made in 0.25 *M* sucrose containing 0.0018 *M* calcium chloride and the nuclei isolated by a modified procedure.[14] Under these conditions, 70 to 90% of the nuclei can be recovered in preparations containing less than 0.5% of the free mitochondria of the homogenate and less than 1% of the cells of the original whole tissue. Unfortunately, however, the calcium-containing sucrose solution is not a suitable medium for the separation of mitochondria and microsomes.

(3) *Isolation of Mitochondria.* The cytoplasmic extract remaining after removal

[60] G. H. Hogeboom, and W. C. Schneider, *J. Biol. Chem.* **197,** 611 (1952).

[61] G. H. Hogeboom and W. C. Schneider, *J. Biol. Chem.* **204,** 233 (1953).

of the nuclear fraction is transferred to Lusteroid centrifuge tubes and centrifuged for 10 minutes at 9200 r.p.m. (5000 *g*) in the International rotor No. 295. The sediment is resuspended in 8.0 ml. of 0.25 *M* sucrose and recentrifuged 10 minutes at 20,800 r.p.m. in the same rotor. At this point the sediment is seen to consist of two definite layers, a lower tan layer and an upper, poorly packed layer of a lighter color. If the livers have not been perfused, the color of the upper layer is a striking pink and easily differentiated from the tan color of the lower layer. The opalescent supernatant fluid, together with the poorly sedimented material, is removed and combined with the supernatant from the first centrifugation. The sediment is resuspended and recentrifuged for 10 minutes at 20,800 r.p.m. A small amount of poorly sedimented material is again obtained and removed along with the clear supernatant fluid. The sediment is made up to a definite volume with 0.25 *M* sucrose and rehomogenized briefly. As indicated by the cytological observations described earlier, this suspension consists of mitochondria essentially uncontaminated by other cellular elements. The yield of mitochondria, as shown by direct count, is approximately 80%.

The layer of poorly sedimented material ("fluffy layer") appearing in the preparation of the mitochondria (see above) requires additional comment. Since microscopic examinations in the authors' laboratory have indicated that this material is largley submicroscopic in character, it has been routinely separated from nitochondria and, by subsequent centrifugation, included in the microsomal fraction. Muntwyler *et al.*[62] independently reached the same conclusions on the basis of the staining characteristics of the fluffy layer. Furthermore, Potter *et al.*[63] have found that, if Janus Green B is added to a tissue suspension and the mixture centrifuged and incubated at 38°, a sharp color boundary occurs between the firmly packed mitochondria, which stain red, and the partially sedimented fluffy layer, which stains blue. The reduction of Janus Green B to yield the red dye, diethylsafranine, is considered to be a specific means of identification of mitochondria. Smellie *et al.*[64] have found in electron microscopic observations both of mitochondria freed of the fluffy layer and of microsomes containing the fluffy layer, that the morphology of the two fractions is distinctly different. On the other hand, Laird *et al.*[65] have claimed that the fluffy layer consists of small mitochondria that can be isolated in purified form. These mitochondria were reported to have a succinoxidase activity equivalent to that of the main mass of mitochondria on the one hand, and to contain a high concentration of PNA, similar to that of microsomes, on the other. Novikoff *et al.*,[66] using the procedure communicated to them by Laird for the isolation of the small "mitochondria," reported that the preparation was, in fact, a mixture of mitochondria and microsomes. Furthermore, Kuff and Schneider in unpublished experiments in the writers' laboratory were unable to obtain any fraction from the fluffy layer with a succinic dehydrogenase activity approaching that of mitochondria, although the fractions were rich in PNA. The correctness of the original conclusion that the fluffy layer is microsomal in its properties, rather than mitochondrial, seems inescapable.

(4) *Isolation of Microsomes.* The supernatants and washings remaining after the

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[62] E. Muntwyler, S. Seifter, and D. M. Harkness, *J. Biol. Chem.* **184,** 181 (1950).

[63] V. R. Potter, R. O. Recknagel, and R. B. Hurlbert, *Federation Proc.* **10,** 646 (1951).

[64] R. M. S. Smellie, W. M. McIndoe, R. Logan, J. N. Davidson, and I. M. Dawson, *Biochem. J.* **54,** 280 (1953).

[65] A. K. Laird, O. Nygaard, and H. Ris, *Cancer Research* **12,** 276 (1952).

[66] A. B. Novikoff, E. Podber, J. Ryan, and E. Noe, *J. Histochem. and Cytochem.* **1,** 27 (1953).

isolation of mitochondria are combined, and 35 ml. is centrifuged for 60 minutes at 25,980 r.p.m. (57,000 *g*) in the type D rotor of the Spinco Model E ultracentrifuge, (Specialized Instruments Corp., Belmont, Cal.). The pellet, which is a transparent amber to red color, depending on whether the livers have been perfused or not, is dispersed by homogenization in 12.5 ml. of 0.25 *M* sucrose, and the suspension is recentrifuged for 30 minutes at 50,740 r.p.m. (148,000 *g*) in the type A preparative rotor. The washed sediment, consisting of microsomes together with about 7% of the original number of mitochondria, is made up to a definite volume by rehomogenization in 0.25 *M* sucrose, and the supernatant is combined with that of the first centrifugation to form the final supernatant or soluble fraction of the cytoplasm.

If the Model L Spinco centrifuge is used in the isolation of microsomes, rotors No. 30 and 40 can be employed, or the entire procedure can be carried out with rotor No. 40. Microsomes can also be sedimented at maximum speed in the multispeed attachment of the International centrifuge. In this case, the time of centrifugation must be prolonged (2 hours or more), and the tubes should have a small diameter (½ to ⅝ inch) to shorten the path of sedimentation. It may also be noted that the initial sedimentation of microsomes results in a firmly packed pellet containing little of the soluble fraction. It is therefore probably not essential to wash the particles by resuspension and resedimentation.

f. Isolation of Pigment Granules

Melanin granules have been isolated by differential centrifugation from amphiuma liver,[67] from the ciliary processes of beef eyes,[68] from frog eggs,[69] and from rat and mouse melanomas.[67,70] The granules have also been obtained recently from melanomas[71] by means of the chromatography of tissue suspensions on Celite columns. Conclusions relating to the biochemical properties of melanin granules must be weighed in terms of possible contamination of the preparations by mitochondria, which are of a similar size. In the case of melanin granules isolated from amphiuma liver, electron micrographs have conclusively demonstrated the absence of mitochondria or other particulate material.[67] Effective separation of the mitochondria and melanin granules of frog eggs was also obtained by Recknagle,[69] who observed striking differences in the cytochrome oxidase activity of the two particulates. On the other hand, the question of mitochondrial contamination of the melanin granules isolated from the other tissues has not been adequately resolved.[67,72] Although electron micrographs of melanin granules isolated by chromotography from pigmented tumors do not disclose the presence of many extraneous elements,[71] the view that the granules carry most of the enzyme systems associated with the mitochondria of other cell types (in contrast to Recknagel's finding[69]) is open to some question. Thus, the specific activities of the mitochondrial enzymes in the pigmented tumors are not

[67] A. Claude, *Harvey Lectures* **43,** 121 (1947–48).

[68] H. Herrmann and M. B. Boss, *J. Cellular Comp. Physiol.* **26,** 131 (1945).

[69] R. O. Recknagel, *J. Cellular Comp. Physiol.* **35,** 111 (1950).

[70] M. W. Woods, H. G. duBuy, D. Burk, and M. L. Hesselbach, *J. Natl. Cancer Inst.* **9,** 311 (1949); H. G. duBuy, M. W. Woods, D. Burk, and M. D. Lackey, *J. Natl. Cancer Inst.* **9,** 324 (1949).

[71] V. Riley, G. Hobby, and D. Burk, *in* "Pigment Cell Growth" (Gordon, ed.), p. 231. Academic Press, New York, 1953.

[72] A. J. Dalton and M. D. Felix, *in* "Pigment Cell Growth," (Gordon, ed.), p. 267. Academic Press, New York, 1953.

known, and it is therefore not possible to determine the extent of mitochondrial contamination that could account for the enzymes supposedly associated with melanin granules. Furthermore, the suggestion that melanin granules are modified forms of mitochondria and that some pigmented cells do not contain unpigmented mitochondria[70] has not received support from more recent studies with the phase contrast and electron microscopes.[72]

The isolation of chloroplasts by differential centrifugation was first reported by Granick.[73] It is of interest that the isolation of these structures was most successful when sucrose solutions were used as the media.

g. Isolation of Secretory Granules

The isolation of secretory granules from liver[74] and pancreas[74,75] was reported by Claude. The identification of the granules from liver as secretory in nature was disputed by Lazarow[76] and Hoerr,[77] who felt that the isolated structures were mitochondria. Although Claude[78] later admitted that a large proportion of the granules was probably mitochondria, he maintained that at least some were secretory granules. Subsequently, Hogeboom *et al.*[19] found that most of the secretory granules of the liver cell apparently did not exist as formed elements when the cells were disrupted. They also found that the isolated mitochondrial fraction was free of secretory granules and that cytoplasmic elements staining with neutral red (e.g., secretory granules and lipid droplets) migrated centripetally when homogenates prepared in sucrose solutions were centrifuged. It would appear from these observations that the isolation of secretory granules in pure form remains to be accomplished.

h. Isolation of Golgi Apparatus

The history of the Golgi apparatus has been fraught with uncertainty because of difficulties in its cytological identification in fresh preparations. A part of the failure to demonstrate the structure in the living cells of all tissues has been the lack of agreement concerning its vital staining properties. On the one hand, it is claimed that the Golgi substance or its precursor is stainable in unfixed cells with neutral red or methylene blue, while on the other, it is claimed that the material is refractory to staining with these dyes. Recently, Worley[79] reported the identification of the Golgi apparatus in the form of droplets that stained with neutral red and were found in the soluble or nonsedimentable fraction of liver homogenates. Identification was based upon the observation that the droplets formed artificial Golgi networks, similar to those seen in fixed preparations of liver on addition of Nile Blue sulphate, but no further attempts were made to separate or characterize the bodies. With regard to the identification of these droplets as the Golgi apparatus on the basis of the neutral red stain, it is necessary to recall that Hogeboom *et al.*[19] also observed the presence of droplets stainable with neutral red in the nonsedimentable fraction of liver. They observed, furthermore, that there were two types of droplets with similar staining properties in the intact unfixed liver cell: one, 0.5 to 1.0 μ in diameter, located at the

[73] S. Granick, *Am. J. Botany* **25**, 558 (1938).
[74] A. Claude, *Cold Spring Harbor Symposia Quant. Biol.* **9**, 263 (1941).
[75] A. Claude, *Biol. Symposia* **10**, 111 (1943).
[76] A. Lazarow, *Biol. Symposia* **10**, 9 (1943).
[77] N. L. Hoerr, *Biol. Symposia* **10**, 185 (1943).
[78] A. Claude, *J. Exptl. Med.* **80**, 19 (1944).
[79] L. G. Worley, *Exptl. Cell. Research* **2**, 684 (1951).

periphery of the cell, and the other, 2 to 3 μ in diameter, located at the interior of the cell. The former were considered to correspond to secretory granules and the latter to lipid droplets that accumulated as the result of fasting. These and other studies[80,81] make it evident that the status of the Golgi apparatus in liver is unsettled—a situation that is likely to persist unless the material can be identified with certainty in the unfixed liver cell.

The existence of a Golgi apparatus in cells of the epididymis, however, appears to be more firmly established. Thus, in investigations reported by Dalton and Felix,[82] examination of unfixed, unstained epididymal cells with phase-contrast illumination revealed a cytoplasmic structure that was as large as the nucleus and in morphological characteristics closely resembled the classical Golgi apparatus visualized in preparations fixed and stained by accepted cytological methods. The structure was refractory to vital staining with neutral red and methylene blue, although numerous granules, staining with these dyes, surrounded it. When epididymal cells were crushed, it was noted that the Golgi substance maintained its characteristic morphology after release from its intracellular environment. Since its morphological integrity was also preserved after the epididymis was homogenized in the apparatus of Potter and Elvehjem,[51] it thus became possible to attempt to isolate the material. Isolation of the Golgi bodies was accomplished[83] by layering 1.4 ml. of a 20 or 25% homogenate of rat epididymis in 0.25 *M* sucrose over a sucrose density gradient and centrifuging for 60 minutes at 35600 r.p.m. (108,000 *g*) in the Spinco SW 39 horizontal rotor. The density gradient was made by pipetting 1.0-ml. layers of 1.11, 0.957, 0.636, and 0.335 *M* sucrose solutions into the centrifuge tube. Each of the sucrose layers contained 0.34 *M* NaCl in addition. After centrifugation, it was found[83] that a layer of fat had migrated centripetally and formed a cap at the top of the tube surmounting a layer containing the soluble proteins of the homogenate. Below the latter, bands of particulate material were seen to occur at the positions corresponding to the junction of the various sucrose layers. The band at the junction of the 0.636 and 0.957 *M* sucrose layers was found to contain most of the Golgi bodies of the original homogenate and, in addition, some submicroscopic particulate material, which was considered to be derived from the Golgi apparatus since it possessed osmication properties characteristic of the Golgi substance. The main mass of submicroscopic particles was found at the bottom of the 0.957 *M* sucrose layer. A sediment at the bottom of the centrifuge tube contained mitochondria, nuclei, sperm, and unbroken cells.

III. Biochemical Properties of Isolated Cell Structures

1. The Nuclear Fraction

As indicated above, the interpretation of analyses of isolated cytoplasmic components is dependent on the analysis of the whole tissue as well as that of all fractions obtainable from the whole tissue. In the initial step in the centrifugal fractionation of homogenates, essentially all of the cell nuclei, together with residual unbroken cells, connective tissue, and some mito-

[80] G. E. Palade and A. Claude, *J. Morphol.* **85,** 35 (1949).
[81] G. E. Palade and A. Claude, *J. Morphol.* **85,** 71 (1949).
[82] A. J. Dalton and M. D. Felix, *Am. J. Anat.* **92,** 277 (1953).
[83] W. C. Schneider, A. J. Dalton, E. L. Kuff, and M. D. Felix, *Nature* **172,** 161 (1953); W. C. Schneider, and E. L. Kuff, *Am. J. Anat.* **94,** 209 (1954).

chondria, are sedimented by low-speed centrifugation, leaving a supernatant containing the cytoplasmic elements of the cell. The sediment has been termed the "nuclear fraction," and because of its heterogeneity is not suitable for detailed studies of the biochemistry of the cell nucleus. Although the latter subject has been discussed at length in Chapter 18, it may be mentioned that analyses of the nuclear fraction have, despite its inhomogeneity, shed considerable light on the localization of enzymes within the nucleus. In the case of practically every enzyme system studied, some activity was recovered in the nuclear fraction. With a few notable exceptions, however, most if not all of the activity could be accounted for by the cytoplasmic elements present. The enzymes that were present in a sufficiently high concentration to indicate actual localization within the cell nucleus were arginase,[2,84] a calcium-activated apyrase[85,86] adenosine-5-phosphatase,[87] and the enzyme catalyzing the synthesis of diphosphopyridine nucleotide (DPN) from ATP and nicotinamide mononucleotide.[60,88] The concentration of the latter enzyme in the nuclear fraction (as well as in essentially homogeneous preparations of isolated nuclei) was unique in that it approached the concentration of DNA itself, suggesting that the synthesis of DPN, at least by this reaction, is an exclusive property of the cell nucleus.

2. The Mitochondrial Fraction

a. Biochemical Data Relating to the Integrity of Isolated Mitochondria

A number of observations have provided evidence that the technique of cell fractionation is a sound tool for studies of the biochemical properties of the mitochondria of the liver cell. One of the most important questions that had to be answered on an experimental basis related to the properties of the mitochondrial membrane. Thus, although the early biochemical investigations of the mitochondrial fraction revealed a clear-cut localization of certain enzyme systems in this cytoplasmic structure, it had to be recognized that these enzymes were "insoluble," i.e, easily sedimented from extracts of practically any tissue, not obtainable in a monodisperse state in true solution, and thus refractive to purification. Notable examples were cytochrome oxidase, succinic dehydrogenase, adenosinetriphosphatase and DPN-cytochrome *c* reductase (Table II). It seemed possible, therefore, despite the cytological evidence for the integrity of isolated mito-

[84] A. H. Schein and E. Young, *Exptl. Cell Research* **3,** 383 (1952).
[85] W. C. Schneider, G. H. Hogeboom, and H. E. Ross, *J. Natl. Cancer Inst.* **10,** 977 (1950).
[86] A. B. Novikoff, L. Hecht, E. Podber, and J. Ryan, *J. Biol. Chem.* **194,** 153 (1952).
[87] A. B. Novikoff, E. Podber, and J. Ryan, *Federation Proc.* **9,** 210 (1950).
[88] G. H. Hogeboom and W. C. Schneider, *Nature* **170,** 374 (1952).

chondria, that the fraction actually consisted of an insoluble residue and that any soluble material present originally in the particles had been extracted during the isolation procedure. (A similar, and still rather controversial, situation exists with respect to cell nuclei isolated in aqueous media (cf. footnotes 60, 88).) During the past three years, however, a considerable amount of experimental evidence has been accumulated in support of the conclusion that the mitochondrial membrane not only is a relatively impermeable structure but remains so after the particles have been isolated in isotonic sucrose solutions. Thus approximately 60% of the total nitrogen of suspensions of isolated mitochondria is released into solution when the particles are disrupted either by exposure to sonic oscillations[89,90] or by other means.[91] Included among these soluble compounds are several enzymes that appear to be localized exclusively in mitochondria. Furthermore, recent investigations have indicated that the membranes of isolated mitochondria are even relatively impermeable to certain polar compounds of low molecular weight. These studies will be discussed in more detail in a later section.

The existence of a mitochondrial membrane thus seems to be definitely established both by the above biochemical investigations dealing with the properties of mitochondria and by numerous cytological studies. The electron micrographs obtained by Palade,[17] for example, clearly demonstrate the presence of a rather thick limiting membrane. The hypothesis advanced by Harmon[57] and by Green[13] that the behavior of mitochondria is compatible with a gel-like structure and does not require the presence of a semipermeable membrane is therefore no longer tenable.

In several investigations, it has been possible to obtain evidence that certain enzymes associated with mitochondria are not present in the fraction as a result of redistribution and adsorption. Thus when intact mitochondria were mixed with solutions of mitochondrial deoxyribonuclease and ribonuclease, and the preparations were then centrifuged, 30% of the deoxyribonuclease and none of the ribonuclease was removed from the solutions by adsorption on the mitochondria.[92] An equivalent amount of microsomes added to the nuclease solution adsorbed 71% of the deoxyribonuclease and 54% of the ribonuclease.[92] Despite the fact that mitochondria were capable of adsorbing some of the deoxyribonuclease, these findings indicated that, if the observed distribution of deoxyribonuclease and ribonuclease in cell fractions had been an artifact due to redistribution and adsorption, both of the enzymes should have been recovered mainly

[89] G. H. Hogeboom and W. C. Schneider, *Nature* **166,** 302 (1950).
[90] G. H. Hogeboom and W. C. Schneider, *J. Biol. Chem.* **194,** 513 (1952).
[91] G. H. Hogeboom and W. C. Schneider, *Science* **113,** 355 (1951).
[92] W. C. Schneider and G. H. Hogeboom, J. Biol. Chem. **198,** 155 (1952).

in the microsomal fraction. Instead, they were largely recovered in the mitochondrial fraction, and it was therefore concluded that their association with mitochondria was not an artifact.[92]

Beinert,[93] in somewhat similar experiments, has shown that the mitochondria of liver homogenates prepared in water are capable of adsorbing large amounts of cytochrome c. When the homogenates were prepared in 0.25 *M* sucrose, however, the mitochondria adsorbed only small amounts of cytochrome c. These results are of interest in view of the previous finding that the cytochrome c of mitochondria was inactive in the succinoxidase system when the particles were isolated from liver homogenates prepared in water[94] but was active when the mitochondria were isolated from homogenates prepared in isotonic saline[94] or in 0.25 *M* sucrose.[58]

b. Biochemical Properties of Isolated Liver Mitochondria

Tables II to IV list the enzymes and related compounds that appear, on the basis of investigations carried out in a number of laboratories, to be localized in mitochondria. In the case of each enzyme or compound studied, values are included indicating both the per cent recovery in the mitochondrial fraction and the concentration in the mitochondria as compared with that in the whole tissue. Specific activities are also included to permit a comparison of the activities of the various enzymes investigated. References to the values in the tables are indicated in the left-hand column, and addtitional experimental work pertinent to the results is referred to in the right-hand column. For reasons discussed above, a considerable mass of data appearing in the literature and obtained in incomplete investigations has not been included. When considered to be of cytochemical significance, the latter studies have served more to confuse than to contribute to the issue. In general, they fall into one or more of four categories: (1) investigations in which enzymes or compounds were found in relatively low concentrations in mitochondria; (2) studies of isolated mitochondria without reference to the whole tissue or to other cell fractions; (3) studies of heterogeneous particulate preparations that include nuclei, nuclear fragments, mitochondria, and microsomes; and (4) experiments in which an enzyme supposedly under study may not have been the rate-limiting component of the reaction mixture. In this connection, it should be pointed out specifically that a number of extremely informative biochemical investigations, aimed at studying the mechanism of complex enzymic reactions rather than the distribution of enzymes within the cell, have been carried out with either isolated mitochondria or heterogeneous particulate preparations as a convenient source of enzyme. The broad cytochemical implications

[93] H. Beinert, *J. Biol. Chem.* **190,** 287 (1951).

[94] W. C. Schneider, A. Claude, and G. H. Hogeboom, *J. Biol. Chem.* **172,** 451 (1948).

TABLE II

BIOCHEMICAL PROPERTIES OF ISOLATED LIVER MITOCHONDRIA

Enzyme system	Recovery (whole tissue = 100)	Concentration (whole tissue = 1)	Specific activity, μM. per hour per mg. of nitrogen	Source of tissue	Additional references and remarks
Cytochrome oxidase[95]	79	3.1	288 (O_2 uptake, 38°)	Mouse	Cf. footnotes 14, 96, 97
DPN-cytochrome c reductase[98]	28	1.2	154 (Cytochrome c reduction, 24°)	Mouse	Cf. footnote 99. Most of remainder in microsomes
TPN-cytochrome c reductase[48]	49	2.3	35 (Cytochrome c reduction, 24°)	Mouse	Also concentrated in microsomes
Succinic dehydrogenase[19]	72	2.8	95 (O_2 uptake, 38°)	Rat	Cf. footnotes 58, 94, 96, 97. Partially inactivated by damage to mitochondria[89]
α-Ketoglutaric oxidase[100]	20	1.5	1.5[a]	Rat	Cf. footnote 101. Mitochondrial activity enhanced by other fractions
Oxalacetic oxidase[102]	45	1.8	18 (O_2 uptake, 38°)	Rat	Mitochondrial activity enhanced by other fractions
Octanoic oxidase[103]	81	2.3	22 (O_2 uptake, 38°)	Rat	Cf. footnote 101. Inactivated by damage to mitochondria[89]
Glutamic dehydrogenase[61]	83	3.3	30 (DPN reduction, 24°)	Mouse	Activity enhanced by damage to mitochondria
Synthesis of ATP[104] (oxidative phosphorylation)	?	?	104 (Phosphate uptake, 28°)	Mouse	Cf. footnotes 100, 105–109. Assay method subject to interference by ATPase
Adenylate kinase[104]	72	?	870 (Loss of ADP, 28°)	Mouse	Cf. footnotes 86, 110
Adenosinetriphosphatase[85]	50	2.2	135 (Phosphate liberated, 38°)	Mouse	Cf. footnotes 66, 86. Activity enhanced by damage to mitochondria[104]
Fumarase[111]	54	2.0	485 (Fumarate formed, 25°)	Mouse	

[a] μM. O_2 per hour per mg. of protein at 37°.

TABLE III

BIOCHEMICAL PROPERTIES OF ISOLATED LIVER MITOCHONDRIA

Enzyme System	Recovery (whole tissue = 100)	Concentration (whole tissue = 1)	Specific activity, μM. per hour per mg. of nitrogen	Source of tissue	Additional references and remarks
Choline Oxidase[112]	78	3.1	8.0 (O_2 uptake, 38°)	Rat	Cf. footnote 113
Betaine Aldehyde Oxidase[114]	50	?	?	Rat	
Tyramine Oxidase[115]	57	2.2	1.9 (NH_3 formed, 20°)	Rat	Cf. footnote 116
Catalase[117]	45	1.8	5.2×10^5 [a]	Rat	Cf. footnote 118. Remainder of activity in soluble fraction
Uricase[119]	65	3.0	6 (uric acid destroyed, 22.5°)	Rat	Cf. footnote 120. Particles containing uricase may not be mitochondria[9,66]
Rhodanese[117]	62	2.5	840 (thiocyanate formed, 20°)	Rat	Cf. footnote 121
Cathepsin[122]	46	2.6	2.9 (Tyrosine liberated, 39°)	Rat	
Glutaminase I[123]	78	3.7	0.56 μM NH_3/mg. of dry weight, 37°	Rat	
Acid Phosphatase[124]	61	?	?	Rat	Cf. footnote 125. Activity enhanced by damage to mitochondria[124]
Ribonuclease[92]	58	2.2	160[b]	Mouse	Activity enhanced by damage to mitochondria
Deoxyribonuclease[92]	73	2.7	29[b]	Mouse	Activity enhanced by damage to mitochondria
Glutamic-oxalacetic transaminase[126]	22	2.3	110 (Glutamic acid formed, 38°)	Rat	
Synthesis of p-aminohippuric acid (PAH)[127]	90	2.8	1.7 (PAH formed, 38°)	Mouse	

[a] μeq. of $NaBO_3$ destroyed per hour per mg. of nitrogen at 37°.

[b] ΔE_{260} of perchloric acid – soluble fragments of DNA or PNA released per hour per mg. of nitrogen at 37.5°.

TABLE IV

BIOCHEMICAL PROPERTIES OF ISOLATED LIVER MITOCHONDRIA

Compound	Recovery (whole tissue = 100)	Concentration (whole tissue = 1)	Specific activity	Source of tissue	Additional references and remarks
Cytochrome c[58]	51	2.3	12.9 μg./mg. of nitrogen	Rat	Cf. footnotes 93, 94. Most of remainder in soluble fraction
Vitamin B_6[128]	33	1.3	0.060 μg./mg. of protein	Rat	
Vitamin B_{12}[129]	56	2.2	0.034 μg./mg. of nitrogen	Mouse	
Riboflavin (Total)[130]	53	1.8	0.30 μg./mg. of protein	Rat	
Flavine adenine[130a] dinucleotide	65	2.3	9.1 μg./mg. of nitrogen	Mouse	
Folinic acid[129]	37	1.8	0.089 μg./mg. of nitrogen	Mouse	
Pantothenic acid[130b]	43	?	?	Rat	
Coenzyme A[130b]	52	?	?	Rat	
Citric acid[131]	67	4.4	55 μg./mg. of nitrogen	Rat	Rat injected with fluoroacetate (3 mg. per kg.)
Phospholipid[97]	27	1.5	7.4 μg. phosphorus/mg. of nitrogen	Rat	Cf. footnote 132

derived from studies on "cyclophorase"[13] are not warranted, on the other hand, because of the cytological heterogeneity of cyclophorase and because most of the conclusions relating to cytochemistry were based on oxygen uptake measurements without reference to the whole tissue[4,5,8,9] (cf. the oxidation of D-isocitrate (Table I)).

[95] W. C. Schneider and G. H. Hogeboom, *J. Natl. Cancer Inst.* **10**, 969 (1950).
[96] G. H. Hogeboom, A. Claude, and R. D. Hotchkiss, *J. Biol. Chem.* **165**, 615 (1946).
[97] W. C. Schneider, *J. Biol. Chem.* **165**, 585 (1946).
[98] G. H. Hogeboom and W. C. Schneider, *J. Natl. Cancer Inst.* **10**, 983 (1950).
[99] G. H. Hogeboom, *J. Biol. Chem.* **177**, 847 (1949).
[100] P. Siekevitz, *J. Biol. Chem.* **195**, 549 (1952).
[101] E. P. Kennedy and A. L. Lehninger, *J. Biol. Chem.* **179**, 957 (1949).
[102] W. C. Schneider and V. R. Potter, *J. Biol. Chem.* **177**, 893 (1949).
[103] W. C. Schneider, *J. Biol. Chem.* **176**, 259 (1948).
[104] W. W. Kielley and R. K. Kielley, *J. Biol. Chem.* **191**, 485 (1951).
[105] J. H. Copenhaver, and H. A. Lardy, *J. Biol. Chem.* **195**, 225 (1952).
[106] A. L. Lehninger, *J. Biol. Chem.* **190**, 345 (1951).
[107] A. L. Lehninger, *in* "Phosphorus Metabolism," (McElroy and Glass, eds.), Vol. 1, p. 344. Johns Hopkins Press, Baltimore, 1951.
[108] A. F. Müller and F. Leuthardt, *Helv. Chim. Acta* **32**, 2349 (1949).
[109] V. R. Potter, G. G. Lyle, and W. C. Schneider, *J. Biol. Chem.* **190**, 293 (1951).
[110] P. Siekevitz and V. R. Potter, *J. Biol. Chem.* **200**, 187 (1953).
[111] E. L. Kuff, *J. Biol. Chem.* **207**, 361 (1954).
[112] C. J. Kensler, and H. Langemann, *J. Biol. Chem.* **192**, 551 (1951).
[113] J. N. Williams, *J. Biol. Chem.* **194**, 139 (1952).
[114] J. N. Williams, *J. Biol. Chem.* **195**, 37 (1952).
[115] G. C. Cotzias and V. P. Dole, *Proc. Soc. Exptl. Biol. Med.* **78**, 157 (1951).
[116] J. Hawkins, *Biochem. J.* **50**, 577 (1952).
[117] S. Ludewig and A. Chanutin, *Arch. Biochem.* **29**, 441 (1950).
[118] H. von Euler and L. Heller, *Z. Krebsforsch.* **56**, 393 (1949).
[119] W. C. Schneider and G. H. Hogeboom, *J. Biol. Chem.* **195**, 161 (1952).
[120] A. H. Schein, E. Podber, and A. B. Novikoff, *J. Biol. Chem.* **190**, 331 (1951).
[121] B. H. Sorbo, *Acta Chem. Scand.* **5**, 724 (1951).
[122] M. E. Maver and A. E. Greco, *J. Natl. Cancer Inst.* **12**, 37 (1951).
[123] J. A. Shepherd and G. Kalnitsky, *J. Biol. Chem.* **192**, 1 (1951).
[124] J. Berthet and C. de Duve, *Biochem. J.* **50**, 174 (1951).
[125] G. E. Palade, *Arch. Biochem. and Biophys.* **30**, 144 (1951).
[126] A. F. Müller and F. Leuthardt, *Helv. Chim. Acta* **33**, 268 (1950).
[127] R. K. Kielley and W. C. Schneider, *J. Biol. Chem.* **185**, 869 (1950).
[128] J. M. Price, E. C. Miller, and J. A. Miller, *Proc. Soc. Exptl. Biol. Med.* **71**, 575 (1949).
[129] M. E. Swendseid, F. H. Bethell, and W. W. Ackermann, *J. Biol. Chem.* **190**, 791 (1951).
[130] J. M. Price, E. C. Miller, and J. A. Miller, *J. Biol. Chem.* **173**, 345 (1948).
[130a] W. C. Schneider and G. H. Hogeboom, unpublished data.
[130b] H. Higgins, J. A. Miller, J. M. Price, and F. M. Strong, *Proc. Soc. Exptl. Biol. Med.* **75**, 462 (1950).
[131] W. C. Schneider, M. J. Striebich, and G. H. Hogeboom, unpublished data.
[132] M. A. Swanson and C. Artom, *J. Biol. Chem.* **187**, 281 (1950).

The mitochondria account for about 25% of the total mass or nitrogen of rat or mouse liver. Their most striking biochemical property (Table II) is their content of enzyme systems related to the respiratory activity of the cell. Thus the terminal respiratory enzyme, cytochrome oxidase, is largely recovered in this cell fraction. That cytochrome oxidase is, in fact, an *exclusive* property of mitochondria has been indicated by the following observations: when liver homogenates were fractionated in isotonic sucrose solutions, 79% of the original cytochrome oxidase activity was recovered in the mitochondria, 20% in the nuclear fraction, and 4% in the microsomes.[95] Although the small amount of activity in the latter fraction was readily explained by contamination with mitochondria, it was necessary, particularly in view of Dounce's conclusion with respect to the cytochrome oxidase of cell nuclei,[1,2] to obtain more complete separation of nuclei and mitochondria in order to determine whether the nuclei themselves contributed to the activity of the nuclear fraction. This was achieved by fractionation of homogenates prepared in isotonic sucrose containing a low concentration of $CaCl_2$.[14] The results of several experiments are summarized in Table V. It can be seen that approximately 90% of the nuclei (as shown by DNA phosphorus determinations) were recovered in a fraction that contained 1.1% of the original number of mitochondria and 1.0% of the original cytochrome oxidase activity. Since, in these and a number of other similar experiments, the cytochrome oxidase activity of preparations of isolated nuclei corresponded closely to the number of mitochondria present, it was concluded that the nuclei themselves did not contain detectable amounts of enzyme activity. The significance of the apparently exclusive localization of cytochrome oxidase in mitochondria becomes evident when it is realized that the ultimate source of energy for all the activities of the cell is mainly provided by this enzymic reaction.

It is not surprising, of course, that systems closely related to cytochrome oxidase should also be found in the mitochondrial fraction. Thus the particles contain a high concentration of cytochrome c (Table IV) and are capable, through the cytochrome c reductases (Table II), of transferring electrons between cytochrome c and the pyridine nucleotides, DPN and TPN. Neither of the cytochrome c reductases is localized exclusively in mitochondria, however, but both are concentrated in the microsomal fraction as well. That mitochondria play an important role in the Krebs cycle series of reactions is indicated by the presence in the fraction of succinic dehydrogenase, which appears to be an exclusive property of mitochondria,[19] fumarase, and by the oxidation of α-ketoglutarate, oxalacetate, and octanoate (Table II). There is, in addition, evidence from *in vivo* experiments that the synthesis of citrate by the condensing enzyme of Stern

TABLE V

CYTOCHROME OXIDASE ACTIVITY OF LIVER FRACTIONS[14]

Preparation	Total nitrogen, per cent of original	Cytochrome oxidase: Per cent of original	Cytochrome oxidase: Concentration (homogenate = 1)	DNA phosphorus: Per cent of original	DNA phosphorus: Concentration (homogenate = 1)	Total number of mitochondria, per cent of original
Homogenate	100[a]	100	1.0[b]	100	1.0[c]	100[d]
Nuclei	13	1.0	0.08	93	7.4	1.1
Mitochondria	39	94	2.7	12	0.3	87
Supernatant (microsomes + soluble fraction)	49	1.5	0.03			

[a] 23.7 mg. of nitrogen per g. of whole tissue.
[b] Specific activity = 4.15 μM. of reduced cytochrome c oxidized per min. per mg. of total nitrogen at 24°.
[c] 9.4 μg. of DNA phosphorus per mg. of total nitrogen.
[d] 11.2 $\times$ 10^{10} mitochondria per g. of whole tissue.

and Ochoa[133] is carried out within the mitochondria. Thus, if fluoroacetate is injected into female rats, a procedure resulting in the accumulation in the livers of relatively large amounts of citric acid, and the livers are then homogenized in sucrose solutions and fractionated in the centrifuge, most of the citric acid is recovered in the mitochondrial fraction (Table IV). The synthesis of citrate by isolated kidney mitochondria has been demonstrated by Kalnitsky.[134] Glutamic dehydrogenase, which is closely related to the Krebs cycle and requires DPN or TPN as a coenzyme, is largely recovered in the mitochondrial fraction (Table II). By means of experiments similar to those described for cytochrome oxidase (Table V), it was possible to demonstrate that glutamic dehydrogenase is solely confined to mitochondria.[61] Although complete data are not available on the intracellular distribution of β-hydroxybutyric dehydrogenase, this DPN-requiring enzyme, according to Lehninger,[107] is also localized in mitochondria.

It would be gratifying from a teleological standpoint if all the enzymes of the Krebs cycle were localized in mitochondria. In spite of statements to this effect (cf. footnote 13), the data indicate that some of these enzymes, isocitric dehydrogenase[48] and aconitase[135] being notable examples, are

[133] J. R. Stern and S. Ochoa, *J. Biol. Chem.* **191,** 161 (1951).
[134] G. Kalnitsky, *J. Biol. Chem.* **179,** 1015 (1949).
[135] A. L. Lehninger, personal communication. Recent work by S. R. Dickman and J. F. Speyer [*J. Biol. Chem.* **206,** 67 (1954)], published since this manuscript went to press, indicate that under certain conditions of enzyme assay, aconitase appears to be concentrated to some extent in the mitochondrial fraction.

localized in other cell fractions and that the Krebs cycle is an integrated function of several cell structures. Additional evidence for the latter view has been provided by the finding that the oxidation of α-ketoglutarate and oxalacetate is stimulated by the addition of other fractions which by themselves are inactive.[100,102]

A reaction that also has an important bearing on the role played by the mitochondrion in the integrated activities of the cell is the synthesis of adenosine triphosphate through oxidative phosphorylation (Table II). In the presence of an oxidizable substrate, such as α-ketoglutarate, glutmate, or succinate, and with added inorganic phosphate, Mg^{++}, and cytochrome c, adenosine diphosphate is rapidly converted to ATP by isolated mitochondria. The presence of adenylate kinase, which establishes an equilibrium among ADP, ATP, and adenosine-5-phosphate, makes it possible for mitochondria also to convert the latter compound to ATP. The complexity of the oxidative phosphorylation reaction and the fact that it is subject to interference by side reactions makes difficult a definitive study of its intracellular distribution. The data of Siekevitz[100] indicate, however, that it may be an exclusive function of mitochondria. A magnesium-activated adenosine triphosphatase, that removes only the terminal phosphate from ATP,[136] is also concentrated in this fraction but, as shown by Kielley and Kielley,[104] is inactive when the mitochondria are freshly isolated in isotonic sucrose solution. The latter investigators have also demonstrated that when the mitochondria are disrupted in a Waring Blendor, the adenosinetriphosphatase is rendered highly active and remains attached to fragments sedimentable only at high centrifugal forces, whereas adenylate kinase passes into solution, and the oxidative phosphorylation reaction disappears.[136] The finding that the adenosinetriphosphatase activity is strongly inhibited by adenosine diphosphate and that mitochondria contain considerable quantities of the latter compound[136] may account, at least in part, for the fact that the intact particles show no adenosinetriphosphatase activity. The means by which the mitochondrion is able to control these three reactions is an interesting problem and may depend on the spatial relationship of the enzyme systems within the particle.

Because of its high energy phosphate bonds, ATP occupies a central position in cellular metabolism, taking part in many reactions that are widely distributed among the various cell structures. ATP has, of course, received considerable attention as a possible source of energy for synthetic reactions. Our present knowledge of the mechanism of biosynthesis of this compound has provided a strong foundation for Claude's suggestion[67] that the mitochondrion is the power plant of the cell.

As shown in Table III, other enzymes apparently associated with mito-

[136] W. W. Kielley and R. K. Kielley, *J. Biol. Chem.* **200**, 213 (1953).

chondria are choline, betaine aldehyde, and tyramine oxidases. According to Claude,[137] D-amino acid oxidase is also concentrated in mitochondria, but complete data supporting this statement have not been published. Dianzani[138] has reported the recovery in mitochondria of 60% of the α-glycerophosphate dehydrogenase of liver homogenates (cf. footnote 137), but the enzyme assay was probably inadequate in that it was based on oxygen uptake determinations without added cofactors such as DPN and cytochrome c. Furthermore, this finding could not be confirmed in unpublished experiments in the writers' laboratory, since the reverse reaction, i.e., the oxidation of reduced DPN in the presence of dihydroxyacetone phosphate, was largely carried out by the soluble fraction of liver homogenates.

Of the other enzymes listed in Table III, several deserve special comment. Recent experiments have suggested that uricase, which is concentrated in both the mitochondrial and microsomal fractions, is associated with particles devoid of respiratory enzyme activity and containing much more PNA than the mitochondrial fraction.[66,139] This finding emphasizes the importance of studying the biochemical homogeneity of the cell fractions, a problem that will be discussed in a later section.

It is rather surprising that ribonuclease and deoxyribonuclease should be concentrated in the mitochondrial fraction, since the native substrates for both of these enzymes are present in other cell fractions. It should be noted, however, that Lang *et al.*,[140] using a viscosimetric method of enzyme assay, have reported that the deoxyribonuclease of kidney is mainly recovered in the nuclear fraction. The determination of the deoxyribonuclease activity concentrated in liver mitochondria was based on the rate of release from highly polymerized DNA of ultraviolet-absorbing fragments soluble in dilute perchloric acid. The possibility must therefore be borne in mind that successive steps in the depolymerization of DNA may be carried out by more than one type of cell structure.

The synthesis of *p*-aminohippuric acid, a reaction somewhat analagous to the synthesis of a peptide bond and studied in detail by Cohen and McGilvery,[141] was found by Kielley and Schneider[127] to be almost entirely localized in mitochondria (Table III). Leuthardt and Nielsen[142] have reported the synthesis of hippuric acid and Muller and Leuthardt[108,143] the synthesis of citrulline by isolated mitochondria.

[137] A. Claude, *Am. Assoc. Advancement Sci. Research Conf. on Cancer* p. 223 (1944).
[138] M. U. Dianzani, *Arch. fisiol.* **50**, 187 (1951).
[139] E. L. Kuff and W. C. Schneider, *J. Biol. Chem.* **206**, 677 (1954).
[140] K. Lang, G. Siebert, I. Baldus, and A. Corbet, *Experientia* **6**, 59 (1950).
[141] P. P. Cohen and R. W. McGilvery, *J. Biol. Chem.* **171**, 121 (1947).
[142] F. Leuthardt and H. Nielsen, *Helv. Chim. Acta* **34**, 1618 (1951).
[143] A. F. Müller and F. Leuthardt, *Helv. Chim. Acta* **32**, 2289 (1949).

As indicated in Table IV, a number of vitamins and coenzymes are concentrated in mitochondria, as well as citric acid, which was mentioned above, and phospholipid. The pentose nucleic acid of mitochondria will be discussed in a later section.

c. Biochemical Properties of the Mitochondria of Tissues Other than Liver.

There is an increasing body of evidence that the morphological similarities of the mitochondria of different tissues[17,18] are paralleled by similarities in biochemical properties. This observation has, in fact, been extended to include even the mitochondria obtained from such widely different sources as unicellular organisms,[40] insects (cf. footnotes 144, 145), plant tissues (cf. footnote 146), and amphibia.[69] Recently, a considerable amount of attention has been given to the properties of the sarcoplasmic granules (or sarcosomes) isolated by centrifugation from mammalian cardiac muscle.[147-151] In general, these preparations are similar to liver mitochondria both in their content of respiratory enzymes and, according to the phase microscopic observations of Harmon and Feigelson,[152] in their morphological characteristics. Cleland and Slater,[150] however, report one pronounced biochemical difference between heart sarcosomes and liver mitochondria: the former contained only 1% of the adenylate kinase activity of the whole tissue, whereas 72% of the adenylate kinase of liver was recovered in the mitochondria.[104] Perry,[153] on the other hand, reported a high adenylate kinase activity in preparations of sarcosomes isolated from skeletal muscle but, as Cleland and Slater[150] have pointed out, did not refer the activity of the sarcosomes to that of the whole tissue and therefore was probably not justified in arriving at cytochemical conclusions. It is readily seen from the data of Table III, in fact, that adenylate kinase is such a powerful enzyme that its presence in trace amounts in a single fraction could be easily misinterpreted in the absence of a complete balance sheet. Cleland and Slater[150] have made an additional important contribution in showing that the well-known Keilin-Hartree heart muscle preparation[154,155] consists

[144] M. I. Watanabe and C. M. Williams, *J. Gen. Physiol.* **34**, 675 (1951).
[145] L. Levenbook, *J. Histochem. and Cytochem.* **1**, 242 (1953).
[146] A. Millerd, and J. Bonner, *J. Histochem. and Cytochem.* **1**, 254 (1953).
[147] G. W. E. Plaut and K. A. Plaut, *J. Biol. Chem.* **199**, 141 (1952).
[148] J. W. Harmon and M. Feigelson, *Exptl. Cell Research* **3**, 509 (1952).
[149] E. C. Slater and K. W. Cleland, *Nature* **170**, 118 (1952).
[150] K. W. Cleland and E. C. Slater, *Biochem. J.* **53**, 547 (1953).
[151] E. C. Slater and K. W. Cleland, *Biochem. J.* **53**, 557 (1953).
[152] J. W. Harmon and M. Feigelson, *Exptl. Cell Research* **3**, 58 (1952).
[153] S. V. Perry, *Biochem. et Biophys. Acta* **8**, 499 (1952).
[154] D. Keilin and E. F. Hartree, *Proc. Roy. Soc. (London)* **B125**, 171 (1938).
[155] D. Keilin and E. F. Hartree, *Biochem. J.* **41**, 500 (1947).

of small particles derived from fragmentation of the sarcosomal membrane. The latter results are strikingly similar to those obtained by the writers in studies of disintegrated liver mitochondria.[89,90]

A few studies of the fractions isolated from brain[156,157] and from kidney[97,102] have indicated that the mitochondria of these tissues are also generally similar in their biochemical properties to the mitochondria of liver. Brody *et al.*,[157] however, obtained a much higher recovery of DPN-cytochrome c reductase in brain mitochondria than was found in the corresponding liver fraction (Table II).

Recently, some very interesting data have been obtained by Watanabe and Williams,[144] Sacktor,[158] and Levenbook[145] in studies of sarcosomes isolated from the flight muscle of certain flies. A review on the subject has been published by Levenbook.[145] These relatively large (2.5 μ in diameter) bodies account for approximately 33% of the entire flight muscle dry weight, can be isolated by a relatively simple procedure, and possess most, if not all, of the cytological characteristics of the mitochondria of the cells of vertebrates. There is, furthermore, a remarkable similarity in the enzymic properties of the insect muscle sarcosomes and of liver mitochondria.[144,145,158] This is particularly evident in Sacktor's[158] investigation of adenosinetriphosphatase and adenylate kinase. His observations are almost identical to those made by Kielley and Kielley[104,135] on isolated liver mitochondria.

As indicated previously, Andresen *et al.*[40] found that succinic dehydrogenase is probably localized in the mitochondria of the amoeba *Chaos Chaos*, and Recknagel[69] found that cytochrome oxidase is concentrated in the mitochondria of frog eggs.

A number of studies dealing with the properties of cell fractions isolated from plant tissues have been published in recent years.[146,159-167] In general, the data suggest that the metabolic role of plant mitochondria is basically similar to that of the mitochondria of animal tissues. It has also been

[156] T. M. Brody and J. A. Bain, *J. Biol. Chem.* **195,** 685 (1952).
[157] T. M. Brody, R. I. H. Wang, and J. A. Bain, *J. Biol. Chem.* **196,** 821 (1952).
[158] B. Sacktor, *J. Gen. Physiol.* **36,** 371 (1953).
[159] H. A. Stafford, *Physiol. Plantarum* **4,** 696 (1951).
[160] J. H. McClendon, *Am. J. Botany* **39,** 275 (1952).
[161] J. H. McClendon, *Am. J. Botany* **40,** 260 (1953).
[162] C. R. Stocking, *Am. J. Botany* **39,** 283 (1952).
[163] P. Saltman, *J. Biol. Chem.* **200,** 145 (1953).
[164] H. G. duBuy, M. W. Woods, and M. D. Lackey, *Science* **111,** 572 (1950).
[165] J. Bonner and A. Millerd, *Arch. Biochem. and Biophys.* **42,** 135 (1953).
[166] A. Millerd, *Arch. Biochem. and Biophys.* **42,** 194 (1953).
[167] A. Millerd, J. Bonner, B. Axelrod, and R. S. Bandurski, *Proc. Natl. Acad. Sci. U. S.* **37,** 855 (1951).

shown that the cytochrome oxidase,[168-171] succinic dehydrogenase,[169] DPN-cytochrome c reductase,[171] and α-glycerophosphate dehydrogenase[171] of yeast cells are associated with sedimentable particles that may be homologues of animal mitochondria. Of interest is the fact that in a mutant strain of yeast cells ("petite colonie"[172]) characterized by the total absence of respiration sensitive to KCN, these particulate-bound enzymes are lost, whereas other enzymes found in the soluble fraction of normal yeast cells are partially or completely retained.[170,171]

d. Biochemical Investigations Relating to the Structural Organization of Mitochondria

A number of interesting properties of mitochondria have been disclosed by biochemical investigations in which the mitochondrial membranes have been either altered by exposure to hypotonic solutions or actually disrupted by mechanical means. When, for example, a suspension of mitochondria is exposed to sonic oscillations (9 kc. per second) at a low temperature for periods up to 30 minutes, essentially all the particles are disrupted, leaving an opalescent, brownish-yellow preparation that is optically empty in the light microscope. If the preparation is centrifuged for 30 minutes at high speed (40,000 to 50,000 r.p.m. in an angle rotor), approximately 60% of the original total nitrogen is recovered in a clear, highly colored supernatant and the remainder in a transparent brownish pellet.[89-91] Examination of the latter particulate material in the analytical ultracentrifuge has revealed that it is polydisperse but does contain several apparently discrete components with sedimentation constants of 25 S. (Svedberg units) or greater,[90] Most of the nitrogen in the supernatant represents proteins having sedimentation constants of from approximately 4 to 12 S.[89,90] A typical refractive index pattern obtained in the analytical ultracentrifuge with the soluble proteins of rat liver mitochondria is shown in Fig. 1A. Much of the total protein appears as a symmetrical peak with a sedimentation constant of 6.3 *S*. In addition, there is a trailing peak (S = *ca.* 4) that appears to be polydisperse, as well as a rapidly sedimenting component (S = *ca.* 12) that is present in relatively low concentration. An identical pattern was obtained with the soluble proteins of mouse liver mitochondria.[91] Attempts to fractionate the mixture at low temperature with ethanol in the presence of buffer at low ionic strength disclosed another component (S = *ca.* 5) that is apparently masked by the main com-

[168] H. Chantrenne, *Enzymologia* **11**, 213 (1943–45).
[169] P. P. Slonimski and B. Ephrussi, *Ann. inst. Pasteur* **77**, 47 (1949).
[170] H. E. Hirsch, *Biochim. et Biophys. Acta* **9**, 674 (1952).
[171] P. P. Slonimski and H. E. Hirsch, *Compt. rend.* **235**, 741 (1952).
[172] B. Ephrussi, *Harvey Lectures* **46**, 45 (1950–51).

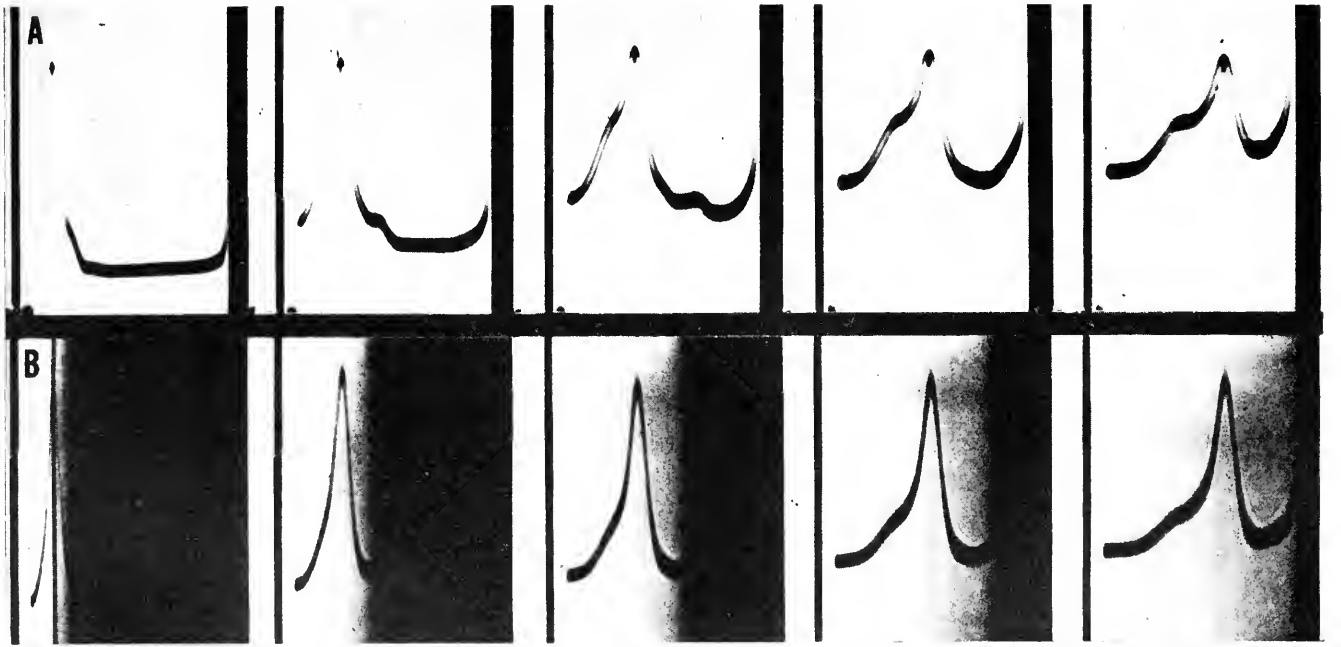

FIG. 1. Sedimentation pattern of mitochondrial proteins. Speed: 59,780 r.p.m. (Spinco Model E Ultracentrifuge, Type A Analytical Rotor); interval between photographs: 16 minutes; rotor temperature: 25°; magnification: 2 diameters. *A*. Unfractionated mixture; photographs taken with monochromatic light at 546 mμ. *B*. Similar preparation after fractionation with ethanol and ammonium sulfate; photographs taken with monochromatic light at 405 mμ; the absorption boundary coincides with the rapidly sedimenting component of *A*.

ponent (S = 6.3). Although the latter protein has not as yet been obtained in a pure state, its behavior on fractionation and the finding that it is precipitated on dialysis against water suggest that it is a type of globulin. Of some interest is the finding that it could not be detected in preparations obtained from the mitochondria of a liver tumor.[91]

Fig. 1*B* shows the sedimentation pattern of a fraction obtained from the mitochondrial proteins by precipitation with 25% ethanol at pH 6.0 and then reprecipitation between 35 and 50% saturation with ammonium sulfate. It can be seen that the main component (S = 6.3) is concentrated to a considerable extent. Since this fraction, like the original preparation, was highly colored and apparently included a heme-containing protein (as indicated by a pronounced absorption peak at 410 mμ), it was desirable to determine what components absorbed light at this wavelength. Accordingly, the photographs of Fig. 1*B* were taken with an interference filter that isolated the mercury arc spectral band at 405 mμ. It can be seen that practically all the absorbing material was associated with the small, rapidly sedimenting peak (S = *ca.* 12). It may be mentioned that this component sediments at approximately the same rate as do purified preparations of catalse (cf. footnote 173).

When disruption of liver mitochondria was accomplished by forcing the particles through a small orifice under high pressure,[91] the protein was strikingly different from that of Fig. 1*A*. Components, usually polydisperse, with sedimentation constants of 4.0, 5.1, 7.5, and 12 were visible, but the main component of Fig. 1*A* (S = 6.3) could not be seen. The results of additional experiments (unpublished) have suggested that the latter protein is either denatured or altered in some other way when the mitochondria are forced through an orifice.

Damage to the mitochondrial membrane either by the above two procedures, by the action of the Waring Blendor, or by lysis of the particles on exposure to distilled water, results in the release of several enzymes into solution. These include acid phosphatase,[124] adenylate kinase,[104,135] glutamic dehydrogenase,[61] fumarase,[111] ribonuclease,[92] and deoxyribonuclease.[92] The experiments of de Duve *et al.*[3] and Berthet and de Duve[124] are of particular interest in this respect. These investigators found that the acid phosphatase of liver mitochondria is inactive when the particles are intact but is rendered highly active by damage to the mitochondrial membrane. These and additional experiments indicated that the membrane is relatively impermeable to the substrate, β-glycerophosphate, and that acid phosphatase is probably present in a diffusible state within the particles. An almost identical situation was found by the writers to hold for glutamic dehydrogenase.[61]

[173] K. Agner, *Biochem. J.* **32,** 1702 (1938).

The probability that the intact mitochondrial membrane is relatively impermeable to compounds of low molecular weight is also brought out by the high recovery of citrate in the liver mitochondria of rats receiving fluoroacetate injections (Table IV). In this respect, it may also be noted that the citrate was retained by the particles when they were isolated and washed in isotonic or hypertonic sucrose solutions but was completely extracted when the particles were washed in water. Bartley and Davies[175] have recently reported observations somewhat along the present lines. Thus isolated kidney mitochondria, when maintained under conditions of active metabolism, were found to contain hydrogen, sodium, potassium, magnesium, and phosphate ions in concentrations 2 to 26 times as great as the corresponding concentrations in the surrounding fluid. The implications of these experiments in terms of renal physiology are, of course, of great interest.

As pointed out earlier, adenosinetriphosphatase is another example of an enzyme that is inactive in intact mitochondria. When the mitochondria are disrupted, however, adenosinetriphosphatase does not pass into solution but remains attached to particles sedimentable by high-speed centrifugation.[136] The tremendous activation of adenosinetriphosphatase resulting from damage to the mitochondria[136] is not readily explained on the ground that the intact membrane is impermeable to ATP, since recent data have indicated that ATP can penetrate the membrane.[110] It is surprising, however, that the membrane should be permeable to ATP and impermeable to such compounds as β-glycerophosphate and citrate.

Several enzyme systems in addition to adenosinetriphosphatase are found mainly in the pellet when preparations of mitochondria, disintegrated either by sonic oscillations or by other means, are clarified by high-speed centrifugation. Among these are succinic dehydrogenase, which is inactivated to a considerable extent by sonic oscillations, cytochrome oxidase, and DPN-cytochrome c reductase.[89] Interestingly enough, cytochrome c, a soluble protein of low molecular weight, is also attached to these sedimentable particles. Table VI shows the distribution of cytochrome oxidase, DPN-cytochrome c reductase, and cytochrome c in fractions obtained from disrupted mitochondria.[90] In these experiments, isolated rat liver mitochondria suspended in 0.25 *M* sucrose were disintegrated by sonic oscillations and centrifuged in the cold at 50,740 r.p.m. (148,000 *g*) for 30 minutes in the Spinco Model E ultracentrifuge (Type A rotor). The pellet was redispersed in 0.25 *M* sucrose and resedimented by 1 hour's centrifugation at the same speed (rapidly sedimenting particles of Table VI). The supernatant from the first centrifugation was centrifuged for 1 hour at 50,740

[174] J. Berthet, L. Berthet, F. Appelmans, and C. de Duve, *Biochem. J.* **50,** 182 (1951).
[175] W. Bartley and R. E. Davies, *Biochem. J.* 52, xx (1952).

TABLE VI

DISTRIBUTION OF CYTOCHROME OXIDASE, DPN-CYTOCHROME C REDUCTASE, AND CYTOCHROME C IN PREPARATIONS OF DISRUPTED MITOCHONDRIA[90]

Preparation	Total nitrogen, per cent of original	Cytochrome oxidase		DPN-cytochrome c reductase		Cytochrome c	
		Per cent of original	Concentration	Per cent of original	Concentration	Per cent of original	Concentration
Disintegrated mitochondria	100	100	1.0[a]	100	1.0[b]	100	1.0[c]
Rapidly sedimenting particles	32	47	1.5	18	0.6	50	1.6
Slowly sedimenting particles	13	29	2.3	37	2.9	18	1.4
Supernatant	50	13	0.3	41	0.9	13	0.3
Supernatant after resedimentation of slowly sedimenting particles	3	1	0.4	8	2.3		

[a] Specific activity = 13.7 μM. of reduced cytochrome c oxidized per min. per mg. of total nitrogen at 24°.
[b] Specific activity = 4.62 μM. of oxidized cytochrome c reduced per min. per mg. of total nitrogen at 24°.
[c] 10.0 μg. of cytochrome c per mg. of total nitrogen.

r.p.m. and the transparent pellet redispersed in 0.25 *M* sucrose and resedimented. Since the resedimentation of the latter slowly sedimenting particles was incomplete, the supernatant from the last centrifugation was also analyzed. It can be seen from the data of Table VI that the rate of sedimentation of the particles containing DPN-cytochrome c reductase was considerably lower than that of particles containing cytochrome oxidase. The distribution of cytochrome c among the fractions was generally similar to that of cytochrome oxidase. The experiments thus suggest that the mitochondrial cytochrome c is tightly bound in its native state to particulate material that contains cytochrome oxidase. Additional evidence for a close structural proximity of cytochrome c, cytochrome oxidase, and succinic dehydrogenase is provided by the finding that the mitochondrial fragments show a high rate of oxygen uptake in the presence of succinate and in the absence of added cytochrome c.[90]

The experiments of Table VI also demonstrate the inadequacy of relatively low speed centrifugation (e.g., 20,000 to 25,000 *g* for 1 to 2 hours) as a means of testing for the "solubilization" of such particulate enzymes as cytochrome oxidase (cf. footnotes 176, 177). Although 43% of the original cytochrome oxidase activity remained in the supernatant after centrifugation at 148,000 *g* for 30 minutes (Table VI), the experiments[90] indicate that the enzyme system was not in true solution but was still bound to complex, polydisperse fragments of mitochondria.

Damage to or disruption of the mitochondrial membranes results in the complete inactivation of certain complex enzyme systems, including those capable of the oxidation of fatty acids[89] and of oxidative phosphorylation.[136] Whether this inactivation can be fully explained by the destruction of ATP through the activation of adenosinetriphosphatase is not entirely clear. In view of the fact that the succinoxidase system, which is not dependent on ATP, is partially inactivated, the possibility remains that the inactivation results from actual physical separation of the components of the multienzyme systems.

It is evident from these experiments that the biochemical organization of the mitochondrion is certainly no less complex than its internal structure. The general picture is that of an osmotically active system, protected from its environment by a relatively impermeable membrane, and containing a high concentration of proteins (including enzymes) and metabolites in a diffusible state. In addition, a number of enzymes appear to be firmly bound to the structural framework of the mitochondrion.

[176] W. W. Wainio, S. J. Cooperstein, S. Kollen, and B. Eichel, *J. Biol. Chem.* **173**, 145 (1948).

[177] B. Eichel, W. W. Wainio, P. Person, and S. J. Cooperstein, *J. Biol. Chem.* **183**, 89 (1950).

It may be noted that the properties of the mitochondrial membrane are of great importance in a consideration of the validity of enzyme assays. Thus in studies of the distribution of enzymes among cell fractions it would now appear to be imperative to determine routinely by disruption of the mitochondria whether the membrane acts as a barrier between substrate and enzyme.

3. The Microsomal Fraction

An event of major importance in both cytology and cytochemistry was Claude's[74] isolation of a particulate fraction from tissue extracts by means of prolonged centrifugation at gravitational forces of the order of 20,000. Although these particles were at first thought to be mitochondria, it soon became apparent that they were too small to be resolved by the light microscope, and they were therefore referred to as submicroscopic particles or microsomes. This cell fraction accounts for a considerable proportion of the total dry weight or nitrogen of most tissues,—as much as 20 to 25% in the case of rat or mouse liver. It was recognized fairly early in semiquantitative analyses of the microsomes that the particles contained comparatively high concentrations of pentose nucleic acid and phospholipid,[53,54] but additional information concerning their biochemical properties has not been available until recently and is still rather meager.

Table VII lists those properties of the microsomal fraction that have been found in a concentration exceeding that in the homogenate. Omachi and coworkers[178] were the first investigators to offer clear-cut evidence that an enzyme, namely, an esterase (methylbutyrase), was associated with the microsomes. Shortly thereafter, the important electron-transporting system, DPN-cytochrome c reductase, was also found to be concentrated in the fraction.[99] Until Hers *et al.*[179] described their work on the specific glucose-6-phosphatase of liver, however, none of the properties studied appeared to be confined exclusively to microsomes. Thus the cytochrome c reductase and uricase were also concentrated in the mitochondrial fraction, PNA and esterase were diffusely distributed among all other cell fractions, and DPN nucleosidase was found in the nuclear and soluble fractions. The recovery of glucose-6-phosphatase in the microsomes, on the other hand, was nearly complete, the small amount of activity remaining in the other fractions being readily explained on the basis of contamination by microsomes. A similar situation appears to hold for triacetic acid lactonase of rat kidney, as reported by Meister.[180] Schotz, Rice, and Alfin-Slater, in a personal com-

[178] A. Omachi, C. P. Barnum, and D. Glick, *Proc. Soc. Exptl. Biol. Med.* **67,** 133 (1948).
[179] H. G. Hers, J. Berthet, L. Berthet, and C. de Duve, *Bull. soc. chim. biol.* **33,** 21 (1951).
[180] A. Meister, *Science* **115,** 521 (1952).

TABLE VII

BIOCHEMICAL PROPERTIES OF MICROSOMAL FRACTION

Enzyme system or compound	Recovery (whole tissue = 100)	Concentration (whole tissue = 1)	Specific activity, μM. per hour per mg. of nitrogen	Source of tissue	Additional references and remarks
Esterase[178]	47	4.2	2200 (CO_2 liberated, 37.5°)	Mouse	Diffusely distributed in all other fractions
DPN-cytochrome c reductase[98]	59	2.5	330 (Cytochrome c reduced, 24°)	Mouse	Cf. footnote 99. Also concentrated in mitochondria (Table II)
TPN-cytochrome c reductase[48]	36	2.0	29 (Cytochrome c reduced, 24°)	Mouse	Also concentrated in mitochondria (Table II)
Glucose-6-phosphatase[179]	88	?	?	Rat	
Lactonase[180] (kidney)	71	3.7	?	Rat	
DPN nucleosidase[181]	50	?	?	Rat	Also present in nuclear and soluble fractions. Overall recovery = 130 to 140%
Uricase[119]	32	1.2	2.6 (Uric acid destroyed, 22.5°)	Rat	See Table II
Pentose nucleic acid[85]	52	2.3	64[a]	Mouse	See Table VIII

[a] μg. of PNA phosphorus per mg. of nitrogen.

[181] S-C. Sung and J. N. Williams, *J. Biol. Chem.* **197,** 175 (1952).

munication to the writers, have reported that the enzyme hydrolyzing cholesterol acetate is localized exclusively in liver microsomes. It is of interest that the latter group of investigators[182] have recovered most of the free cholesterol of liver in the microsomal fraction and all of the esterified cholesterol in a lipid fraction that migrates centripetally in the centrifuge.

Although other complete studies of the distribution of lipids in liver homogenates apparently have not been reported, investigations of individual fractions have indicated that the microsomes contain much higher concentrations of lipid than does any other cell fraction.[54,183-185] Approximately 40% of the dry weight of microsomes is comprised of lipid, which is mainly in the form of phospholipid. The fact that liver microsomes are pigmented, showing a distinctly reddish hue, has also interested a number of investigators. Bensley[186] first suggested that the pigment represents the products of oxidation of unsaturated lipids, particularly phospholipids, but later[187] stated that the microsomal color is due to adsorbed hemoglobin. Strittmatter and Ball,[188] however, have presented evidence indicating that it is a protoporphyrin hemochromagen resembling but nevertheless distinct from cytochromes a, b, and c.

Obviously, it is not at present possible to present anything like a complete picture of the role of microsomes in cellular metabolism. Except for their apparent ability to transfer electrons between cytochrome c and the pyridine nucleotides, the particles evidently do not play an important part in oxidative or respiratory metabolism. The high PNA content of the fraction and an apparent relationship between PNA and protein synthesis (Chapter 28) have, however, led to some speculation concerning the possible role of microsomes in the synthesis of proteins. In this respect, more recent *in vivo* experiments have shown that after injection of labeled amino acids, the proteins of microsomes show a higher specific activity than do the proteins of any other fraction isolated from liver.[189,190] Siekevitz[100] has extended these observations by showing that, when respiring liver homogenates are incubated in the presence of alanine-C^{14}, both the rates of incorporation and the total incorporation of the amino acid are much higher in microsomes subsequently isolated from the reaction mixture than

[182] M. C. Schotz, L. I. Rice, and R. B. Alfin-Slater, *J. Biol. Chem.* **204,** 19 (1953).
[183] G. L. Ada, *Biochem. J.* **45,** 422 (1949).
[184] C. P. Barnum and R. A. Huseby, *Arch. Biochem. and Biophys.* **19,** 17 (1948).
[185] R. A. Huseby and C. P. Barnum, *Arch. Biochem. and Biophys.* **26,** 187 (1950).
[186] R. R. Bensley, *Anat. Record* **98,** 609 (1947).
[187] R. R. Bensley, *J. Histochem. and Cytochem.* **1,** 179 (1953).
[188] C. F. Strittmatter and E. G. Ball, *Proc. Natl. Acad. Sci. U. S.* **38,** 19 (1952).
[189] E. B. Keller, *Federation Proc.* **10,** 206 (1951).
[190] N. D. Lee, J. A. Anderson, R. Miller, and R. H. Williams, *J. Biol. Chem.* **192,** 733 (1951).

in any other fraction. He has also shown that incorporation does not occur when microsomes or mitochrondria are incubated separately with the substrate, but it does occur when the two fractions are combined. A requirement for the reaction is the addition of substrates and cofactors permitting the simultaneous occurrence of oxidative phosphorylation. During this oxidative reaction, the mitochondria apparently produce a soluble factor, possibly a derivative of ATP, that enables the microsomes to incorporate alanine. These very interesting experiments thus suggest that microsomes contain the enzyme system responsible for the incorporation of the amino acid but in order to carry out the reaction require a source of energy which in this case is provided by the respiratory enzyme systems of the mitochondrion. These findings may well represent another example of the integrated activities of several cell structures in carrying out a complicated metabolic process. There are, in addition, data indicating that microsomes play some role in Krebs cycle reactions,[102] perhaps through their content of the cytochrome c reductases, and in glycolysis.[191]

There is, in addition to the cytological evidence discussed earlier, a considerable amount of biochemical data indicating that microsomes are a group of distinct cellular entities. In this respect, the conclusion of Harmon[57] and Green[13] that microsomes are fragments of mitochondria is not supported by data obtained in a number of other laboratories. There is good reason to believe, in fact, that *qualitative* biochemical differences exist between these two particulate elements of the cell. When adequate fractionation of cytoplasmic components is achieved, glucose-6-phosphatase is recovered almost completely in microsomes.[179] Conversely, the specific cytochrome oxidase activity of the mitochondrial fraction is almost 20 times that of the microsomes,[95] and the low activity of the latter fraction can be readily explained on the basis of the presence of contaminating mitochondria. Furthermore, mitochondrial fragments, although similar in sedimentation characteristics to microsomes, are entirely different in certain biochemical properties. Thus, when isolated mitochondria are disrupted by means of sonic vibrations to yield optically empty suspensions, the mitochondrial fragments isolated by high-speed centrifugation show an even higher specific cytochrome oxidase activity than do the original preparations of intact mitochondria.[89,90] It seems likely that the inability of Green[13] to distinguish biochemically between microsomes and mitochondria results from his use of the Waring Blendor in the preparation of homogenates. Since it is well known that this method of homogenization results in the disruption of a significant proportion of the mitochondria,[8,63,136] the microsomal fraction isolated from such homogenates would be expected to contain mitochondrial fragments and thus to exhibit quali-

[191] G. A. LePage and W. C. Schneider, *J. Biol. Chem.* **176**, 1021 (1948).

tatively the enzyme activities characteristic of the latter particles. In the face of these observations, it is apparent that the hypothesis that microsomes are artifacts derived from mitochondria must involve some rather quaint assumptions.

4. The Soluble Fraction

After the removal from the liver homogenate of nuclei, mitochondria, and microsomes, there remains a supernatant accounting for about 40% of the original total nitrogen. Most of the nitrogen of this "soluble fraction" of the cell is in the form of proteins in solution. In addition, lipids are present that migrate centripetally in the centrifuge,[19,182] but there is apparently little other material that is not in true solution. Although it has been assumed for operational purposes that the soluble fraction represents the "cell sap," the possibility must also be recognized that it may include substances extracted from the particulate elements of the cell. The latter consideration imparts a definite risk to any unequivocal conclusions concerning the cytochemical significance of biochemical analyses of the supernatant. Nevertheless, in view of evidence indicating that soluble proteins are *not* extracted under proper conditions from such structures as nuclei[88,92] and mitochondria (see above), it is probable that analyses of the soluble fraction are in many instances significant from the cytochemical standpoint. As in studies of the biochemical properties of the other isolated cell constituents, however, all conclusions must be carefully weighed in terms of the concentration and recovery obtained.

The presence in the supernatant of a large number of proteins is demonstrated by the complicated patterns obtained in electrophoretic studies by Sorof and Cohen[192,193] and Gjessing *et al.*[194] That a correspondingly large number of enzymes should have been found in the fraction is, of course, not surprising. Perhaps the best example of the role of the soluble fraction in a metabolic process of major importance is found in the studies of LePage and Schneider[191] of anaerobic glycolysis. These investigators followed the rate of lactic acid formation in an anaerobic system including glucose, hexose diphosphate, pyruvate, ATP, and DPN, and found that over 50% of the activity of rabbit liver homogenates could be recovered in the soluble fraction. The other cell fractions showed little or no activity by themselves but were capable of stimulating the activity of the supernatant. Although the complexity of the reaction is such that the rate-limiting component is not known, the implication of this investigation is that the supernatant contains most of the enzymes involved. The enhancement of lactic acid

[192] S. Sorof and P. P. Cohen, *J. Biol. Chem.* **190,** 303 (1951).
[193] S. Sorof and P. P. Cohen, *J. Biol. Chem.* **190,** 311 (1951).
[194] E. C. Gjessing, C. S. Floyd, and A. Chanutin, *J. Biol. Chem.* **188,** 155 (1951).

formation by the particulate fractions is not readily explained but probably comprises another example of the integrated activity of several cell structures in carrying out a complicated metabolic process. A complete picture of the intracellular locale of glycolytic enzymes will be available, however, only when studies are made of the distribution among cell fractions of each individual enzyme. Some recent steps in this direction all lend support to the view that glycolysis is largely a function of the soluble fraction of the cell. Thus Hers, de Duve, and their collaborators[179,195] have shown that hexokinase, phosphorylase, and phosphoglucomutase, and Kennedy and Lehninger[101] that aldolase, are mainly recovered in this fraction. Evidence that lactic dehydrogenase can also be included in the group of nonparticulate enzymes has been obtained in unpublished experiments in the writers' laboratory. It may be mentioned, however, that Crane and Sols[196] have found that a significant proportion of the hexokinase of homogenates of various tissues is sedimented at 18000 g. The recovery of hexokinase in this mixed particulate fraction ranged from 35 % in the case of liver to over 90 % in the case of brain. A somewhat similar situation apparently exists in certain plants.[163]

The soluble fraction of the liver cell, through its content of isocitric dehydrogenase[48] (Table I) and aconitase,[135] apparently takes part in the reactions of the Krebs cycle. The presence in the fraction of 90 % or more of the adenosine deaminase and nucleoside phosphorylase activity of liver homogenates[119] also demonstrates a probable role in the metabolism of purines. The occurrence of certain other enzymes and compounds, including acetylase,[197] glyoxalase,[198] xanthine oxidase,[199] hexose diphosphatase,[179] alkaline phosphatase,[80] and cytochrome c,[94] as well as the numerous instances in which the supernatant has been used as the starting material in the purification of enzymes, attest further to the biochemical complexity of the fraction.

5. On the Biochemical Homogeneity of Isolated Cytoplasmic Structures

It is evident from the observations described above that conclusions as to the localization of biochemical properties in specific cell structures are in most instances based on cytological determinations of the homogeneity of isolated fractions. That enzymes or other compounds could be confined to a single particulate component was challenged by the experiments of

[195] C. de Duve, H. G. Hers, and J. Berthet, *Ind. chim. belge* **17,** 143 (1952).
[196] R. K. Crane and A. Sols, *J. Biol. Chem.* **203,** 273 (1953).
[197] J. Chauveau and L. V. Hung, *Compt. rend.* **235,** 1248 (1952).
[198] E. Kun, *Euclides* **10,** 251 (1950).
[199] V. Meikleham, I. C. Wells, D. A. Richert, and W. W. Westerfeld, *J. Biol. Chem.* **192,** 651 (1951).

Chantrenne,[200] who prepared cytoplasmic extracts of mouse liver and centrifuged them at speeds arbitrarily selected to yield 5 fractions of particles with different sedimentation rates. Analysis of the fractions revealed that their content of PNA, phospholipid, and phosphatases was different and that none of the properties studied was a sole constituent of a single fraction. The results suggested, in fact, that the cytoplasm consists of a continuous spectrum of particles, heterogeneous with respect to size, chemical composition, and enzymic pattern. One objection to Chantrenne's experiments is that a salt solution was used in the preparation of the extracts, and that most of the mitochondria were therefore probably lost as a result of aggregation and sedimentation on removal of nuclei and intact cells. Another shortcoming of the experiments, which was recognized by Chantrenne, lay in the fact that the *total* quantity of enzyme or chemical compound in each fraction was not determined. Furthermore, subsequent work has shown that none of the properties studied is localized exclusively in a single cell structure. These considerations, together with the probability that several of the fractions were mixtures of mitochondria and microsomes, make it evident that the experiments could indicate a greater degree of heterogeneity than actually existed. The objections to Chantrenne's investigation were avoided by Novikoff *et al.*,[66] who fractionated liver homogenates prepared in 0.25 *M* sucrose. The latter workers isolated 8 cytoplasmic fractions and found that those composed mainly of mitochondria exhibited minor degrees of heterogeneity while the microsomal fractions were markedly heterogeneous.

That submicroscopic particles are heterogeneous had already been indicated by the work of Barnum and Huseby,[184] who studied the chemical composition of two sedimentable fractions obtained from liver extracts after removal of mitochondria and larger elements. In this respect, it has been observed in the writers' laboratory that similar cytoplasmic extracts of liver, when examined in the analytical ultracentrifuge or the electrophoresis apparatus, yield refractive index patterns indicating the presence of several classes of particles, differing both with respect to sedimentation rate and electrical charge (unpublished experiments). Petermann and Hamilton[201] and Petermann *et al.*,[202] in a similar study of the sedimentation pattern of submicroscopic particles obtained from liver and from normal and leukemic spleen, report the presence of 5 to 7 groups of particles with characteristic sedimentation rates.

A recent investigation by Kuff and Schneider[9,139] suggests that the

[200] H. Chantrenne, *Biochem. et Biophys. Acta* **1,** 437 (1947).

[201] M. L. Petermann and M. G. Hamilton, *Cancer Research* **12,** 373 (1952).

[202] M. L. Petermann, N. A. Mizen, and M. G. Hamilton, *Cancer Research* **13,** 372 (1953).

mitochondrial fraction of liver may be more heterogeneous biochemically than previous results have indicated. In these experiments, the approach was somewhat different from that of Chantrenne and Novikoff *et al.* The mitochondrial fraction was first isolated by the usual procedure, and then particles in the fraction of different specific gravities were segregated by high-speed centrifugation in a sucrose density gradient. It was found that about 75% of the total nitrogen was recovered in two fractions, in which the concentration of PNA and of several enzymes was almost the same. The finding of greatest interest, however, was that a major part of the uricase and deoxyribonuclease activities of the original preparation of mitochondria was associated with particles accounting for only 10% of the total nitrogen and containing a very low concentration of succinic dehydrogenase. With respect to uricase and succinic dehydrogenase, these results are generally similar to the data reported by Novikoff *et al.*[66] The latter investigators considered the uricase-containing particles to be microsomal in nature. Independent evidence bearing on the question of the biochemical heterogeneity of mitochondria has come from experiments in which a study was made of the livers of rats fed the noncarcinogenic derivative of butter yellow, 2-methyl-4-dimethylaminoazobenzene.[203,204] It was found that the addition of this compound to the diet resulted in a 2.5-fold increase in the number of mitochondria per unit weight of liver. These mitochondria were normal with respect to succinoxidase content, the activity of this enzyme system having increased to the same extent as did the number of mitochondria, but were deficient in a number of other enzymatic activities studied (octanoic acid oxidase, DPN-cytochrome c reductase, uricase, deoxyribonuclease, and ribonuclease). Other investigations have indicated that liver mitochondria can also undergo alterations as a result of hormonal imbalances. The unpublished experiments of Dr. S. H. Wollman of the National Cancer Institute, for example, suggest that the livers of hyperthyroid rats contain a normal number of mitochondria characterized by a greatly increased ability to oxidize succinic acid, while the livers of diabetic rats contain an increased number of mitochondria having the same succinoxidase activity as do normal liver mitochondria. The results with the diabetic and hyperthyroid livers need to be substantiated, however, by actual counts of the number of liver mitochondria.

A conclusive demonstration that mitochondria are biochemically heterogeneous will, if obtained, pose several interesting and important questions. In the first place, would such a finding mean that the mitochondria in each liver cell show varying degrees of differentiation or that the mitochondria

[203] M. J. Striebich, E. Shelton, and W. C. Schneider, *Cancer Research* **13**, 279 (1953).
[204] W. C. Schneider, G. H. Hogebroom, E. Shelton, and M. J. Striebich, *Cancer Research* **13**, 285 (1953).

in one liver cell differs from those in neighboring cells? The answers to questions of this type obviously would depend upon methods more refined than the present cell fractionation technic. If differences do exist between the mitochondria in the same or adjacent cells, it would seem likely that some differences in the microscopic appearance of these granules should have been noted. Perhaps, with the biochemical results as a stimulus, it may become possible to demonstrate cytologically that differences in mitochondria exist and to establish where the variations occur. The biochemical studies may also provide a basis for the study of mitochondrial duplication, both with respect to the mechanism of reproduction of the granules themselves and also with regard to the synthesis of the enzymes and other compounds residing in the mitochondria.

6. The Pentose Nucleic Acid of Isolated Cell Fractions

Deoxypentose nucleic acid (DNA) does not occur normally in the cytoplasm. Although small amounts of DNA have occasionally been found in isolated mitochondria and microsomes, its occurrence in these fractions probably results either from the rupture of some nuclei during the preparation of homogenates[60] or from inadequacies in the method of determining DNA.[63]

The presence of PNA in mitochondria and microsomes was first suggested by Claude's[54] determinations of the ultraviolet absorption spectra of extracts of the particles. His measurements did not, however, distinguish between PNA and other compounds with similar absorption characteristics. Quantitative studies of the distribution of PNA in liver fractions showed that only small amounts were present in the isolated nuclear and mitochondrial fraction and that the major proportion was associated with the unfractionated supernatant remaining after removal of these two particu-

TABLE VIII

Pentose Nucleic Acid Content of Mouse Liver Fractions[85]

Preparation	PNA phosphorus		
	Total,[a] μg.	Recovery, per cent	Concentration, μg. per mg. of nitrogen
Homogenate	92.9	100	28.1
Nuclear fraction	10.2	11.0	19.2
Mitochondria	15.7	16.8	18.4
Microsomes	48.7	52.4	64.2
Soluble fraction	15.3	16.5	12.0

[a] Per 100 mg. of whole tissue or an equivalent amount of each fraction.

late structures.[97] Subsequent experiments, in which improved procedures were used for the fractionation of liver homogenates, have demonstrated that about 50% of the PNA of liver is associated with the isolated microsomes (Table VIII). This fraction is the only one in which the concentration of PNA exceeds that in the original tissue.[19,85,102,103]

On a number of occasions, the question has arisen as to whether the PNA of the nuclear, mitochondrial, and soluble fractions might be a reflection of contamination by microsomes. That cell nuclei contain PNA has been firmly established, however, through several independent lines of evidence. Thus, *in vivo* studies of the rate of incorporation of isotopes have demonstrated that the turnover of nuclear PNA is much more rapid than that of the PNA of any other cell fraction.[64,65,205] Furthermore, there are striking differences in the nucleotide composition of nuclear and cytoplasmic PNA.[206,207] This question is discussed further in Chapters 26 and 28.

The significance of the presence of PNA in the mitochondrial fraction of liver is still open to some question. Attempts to remove the PNA by repeated sedimentation of mitochondria have been unsuccessful,[19] however, and further fractionation both of mitochondria[139] and of homogenates[66] has failed to provide evidence that the PNA of the fraction is associated with extraneous elements. Equivocal results have been obtained in comparisons of the composition of mitochondrial PNA with the composition of the PNA of other fractions. Crosbie *et al.*[206] have found essentially no differences, whereas Elson and Chargaff,[207] in a preliminary report, have noted dissimilarities in nucleotide content. The low concentration of PNA in mitochondria and the possibility of contamination of the preparations by other PNA-containing structures have led the latter investigators to express some reservations as to the significance of their results. Smellie *et al.*[64] have reported essentially no differences in the rate of incorporation of P^{32} in mitochondrial and microsomal PNA obtained from normal or regenerating liver. Khesin,[208] on the other hand, has found that the rate of incorporation in normal liver is slower for mitochondrial than for microsomal PNA but that in regenerating liver the mitochondrial PNA shows a more rapid turnover than does the PNA of any other cytoplasmic fraction.

An appreciable amount of PNA of liver is present in the soluble fraction (Table VIII). That this "soluble" PNA is different from the PNA of the other liver fractions is indicated by the fact that its rate of turnover is second only to that of the nuclear PNA (cf. footnotes 63, 64, 208). The

[205] A. Marshak and F. Calvet, *J. Cellular Comp. Physiol.* **34,** 451 (1949).
[206] G. W. Crosbie, R. M. S. Smellie, and J. N. Davidson, *Biochem. J.* **54,** 287 (1953).
[207] D. Elson and E. Chargaff *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), p. 329. Johns Hopkins Press, Baltimore, 1952.
[208] R. V. Khesin, *Biokhimiya* **17,** 664 (1952).

nucleotide composition of the PNA of the soluble fraction is essentially the same as that of the PNA of other cytoplasmic fractions.[206,207]

Although it is evident that a considerable amount of information is available concerning the intracellular distribution of PNA and the composition of the PNA of various cell fractions, the significance of these results remains uncertain in the absence of definitive data relating to the metabolic function of PNA. The fact that certain viruses are similar to microsomes in their high PNA content (and general physical properties) apparently initiated the suggestion that the latter particles may be the center of protein synthesis within the cell. Although this hypothesis has received some experimental support,[100] the analogy on which it was based does not seem to hold. Thus in certain tissues other than liver (e.g., adult kidney[102] and spleen[209] and embryonic tissues[210]), PNA is associated mainly with fractions that are nonmicrosomal in sedimentation characteristics. Furthermore, studies of PNA turnover demonstrate merely that microsomal PNA is renewed relatively slowly[64,208] and thus fail to reveal any clue as to its metabolic role. The recent experiments of Binkley,[211] indicating that a preparation of protein-free ribose polynucleotide can catalyze an enzymic reaction, offer the greatest promise toward an eventual understanding of the function of the PNA of cell structures.

[209] M. L. Petermann and E. J. Mason, *Proc. Soc. Exptl. Biol. Med.* **69**, 542 (1948).
[210] J. Brachet and R. Jeener, *Enzymologia* **11**, 196 (1944).
[211] F. Binkley, *Exptl. Cell Research* Suppl. 2, 145 (1952).

CHAPTER 22

Biosynthesis of Pentoses

GERTRUDE E. GLOCK

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I. Introduction

The widespread distribution of the pentose sugars in Nature and the important part they play as structural units of many essential cellular constituents such as nucleic acids and coenzymes, makes the question of their origin of considerable interest. Various methods of biosynthesis have been suggested, but, according to our present state of knowledge, only two of these appear to be of general biological significance. These are the hexosemonophosphate oxidative pathway of carbohydrate metabolism (also known as the "direct oxidative pathway" and the "hexosemonophosphate shunt") and the enzymic condensation of C_2 and C_3 compounds. The oxidative pathway leads to the formation of D-ribose-5-phosphate from D-glucose-6-phosphate, whereas the condensation of C_2 and C_3 compounds yields both pentoses and deoxypentoses and appears to be the only known method of biosynthesis of deoxyribose.

II. The Hexosemonophosphate Oxidative Pathway as a Source of Pentose Phosphate

1. Early Investigations

The existence of a pathway, distinct from the glycolytic route, for the oxidation of glucose-6-phosphate was first demonstrated in yeast extracts by Warburg and his co-workers.[1,2] They found that the oxidation of hexosemonophosphate by "Zwischenferment" (now called glucose-6-phosphate dehydrogenase) was triphosphopyridine nucleotide (TPN) specific and, using a purified dehydrogenase preparation,[3] identified the oxidation product as 6-phosphogluconate.[2] Flavoprotein oxidase ("old yellow enzyme"), discovered[1] and isolated[4] in connection with these experiments, was used to reoxidize the reduced TPN. Further oxidation of 6-phosphogluconate by yeast enzymes was also found to be coupled with TPN.[5] Lipmann,[6] working with yeast macerates, suggested that 2-ketophosphogluconic acid would be the first oxidation product and considered that this would yield arabinose-5-phosphate on decarboxylation. Dickens,[7,8] however, found that this pentose phosphate was neither oxidized nor fermented by yeast extracts at a rate sufficient for it to be an intermediate in this pathway, whereas D-ribose-5-phosphate was entirely suitable in this re-

[1] O. Warburg and W. Christian, *Biochem. Z.* **254**, 438 (1932).
[2] O. Warburg, W. Christian, and A. Griese, *Biochem. Z.* **282**, 157, (1935).
[3] E. Negelein and W. Gerischer, *Biochem. Z.* **284**, 289 (1936).
[4] O. Warburg and W. Christian, *Biochem. Z.* **266**, 377 (1933).
[5] O. Warburg and W. Christian, *Biochem. Z.* **287**, 440 (1936).
[6] F. Lipmann, *Nature* **138**, 588 (1936).
[7] F. Dickens, *Biochem. J.* **32**, 1615 (1938).
[8] F. Dickens, *Biochem. J.* **32**, 1626 (1938).

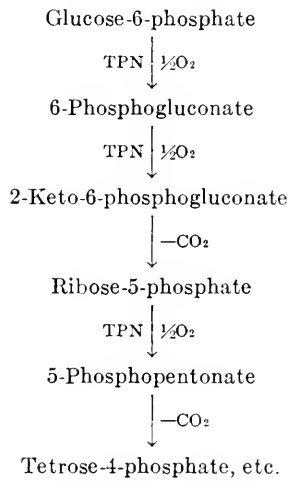

FIG. 1. Dickens' scheme for oxidation of glucose-6-phosphate by yeast enzymes.

spect. It was suggested that during the oxidation of 6-phosphogluconate the necessary change of configuration occurred at carbon atom 2 of the pentose to give D-ribose-5-phosphate. Isolation of the oxidation products of 6-phosphogluconate was attempted by both Dickens[7] and Warburg and Christian[9] and evidence obtained of the formation by successive oxidations and decarboxylations of C_5, C_4, and C_3 phosphates, but none of these was identified. One of the C_5 compounds[7] gave a strong pentose color reaction. Dickens' scheme for the sequence of events in this pathway is given in Fig. 1.

2. Recent Work on the Identification of Pentose Phosphates

a. Ribose-5-phosphate

The possible physiological significance of this oxidative pathway was apparently not appreciated by the early workers in this field since no further systematic work was done for over ten years when Scott and Cohen reinvestigated Dickens' yeast system. Their primary interest was in the origin of ribose and deoxyribose for incorporation into nucleic acids and in the nature of the diverted metabolism in virus-infected bacteria.[10] Using a crude yeast enzyme preparation,[11] the products of enzymic degradation of 6-phosphogluconate were isolated, fractionated, and analyzed,[12] pentose

[9] O. Warburg and W. Christian, *Biochem. Z.* **292,** 287 (1937).

[10] S. S. Cohen, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 35 (1947); *J. Biol. Chem.* **174,** 281 (1948); *Bacteriol. Revs.* **13,** 1 (1949); **15,** 131 (1951).

[11] F. Dickens and H. McIlwain, *Biochem. J.* **32,** 1615 (1938).

[12] D. B. M. Scott and S. S. Cohen, *Science* **111,** 543 (1950); *J. Biol. Chem.* **188,** 509 (1951); *J. Cellular Comp. Physiol.* **38,** Suppl. 1, 173 (1951).

phosphates and pentoses being detected by paper chromatography and ribose and arabinose determined quantitatively by the use of pentose adapted *E. coli*.[13] Both D-ribose and D-arabinose were detected and accounted for 25% and 10%, respectively, of the total apparent pentose present. Of the pentose phosphates, two behaved like ribose-5-phosphate and arabinose-5-phosphate but most was in the form of an unknown pentose phosphate with a characteristic peak at 450 mμ in the Bial reaction. This was considered to be a 1,2-enediol pentose-5-phosphate and was suggested as the primary decarboxylation product of 6-phosphogluconate. Recently, however, Cohen has shown that this unknown pentose phosphate is probably an alkaline degradation product of ribulose-5-phosphate.[14] The following pentose phosphate interrelationships were suggested:[12]

D-arabinose-5-phosphate $\rightleftharpoons$ D-ribulose-5-phosphate $\rightleftharpoons$ D-ribose-5-phosphate

b. Ribulose-5-phosphate

The nature of the intermediates in 6-phosphogluconate oxidation has also been investigated by Horecker and his co-workers[15,16] using a purified yeast dehydrogenase preparation.[17] In order to accumulate the oxidation product without adding stoichiometric quantities of TPN, reoxidation of reduced coenzyme was effected by adding an excess of pyruvate and lactic dehydrogenase. By this means 6-phosphogluconate was oxidized quantitatively to pentose phosphate with catalytic amounts of TPN:

(1) 6-phosphogluconate + $TPN^+ \rightarrow$ pentose-5-phosphate + CO_2 + TPNH + H^+
(2) pyruvate + TPNH + $H^+ \rightarrow$ lactate + TPN^+

Two pentose phosphates were detected which were separated by ion-exchange chromatography on Dowex 1 formate. One phosphate was dextrorotatory and the other lævorotatory, the proportion of each component depending on the time of incubation. The dextrorotatory compound was identified by chromatographic and chemical methods as D-ribose-5-phosphate, the nature of the pentose being confirmed by the preparation of the benzylphenylhydrazone. The lævorotatory compound was obviously a precursor of ribose-5-phosphate since it was the major component early in the reaction but was gradually replaced by ribose-5-phosphate as the incubation proceeded. The pentose of this component was identified as D-ribulose by means of its *o*-nitrophenylhydrazone, and on the basis of its conversion to ribose-5-phosphate was presumed to be ribulose-5-phosphate. An equi-

[13] S. S. Cohen and R. Raff, *J. Biol. Chem.* **188,** 501 (1951).
[14] S. S. Cohen, *J. Biol. Chem.* **201,** 71 (1953).
[15] B. L. Horecker and P. Z. Smyrniotis, *Arch. Biochem.* **29,** 232 (1950).
[16] B. L. Horecker, P. Z. Smyrniotis, and J. E. Seegmiller, *J. Biol. Chem.* **193,** 383 (1951).
[17] B. L. Horecker and P. Z. Smyrniotis, *J. Biol. Chem.* **193,** 371 (1951).

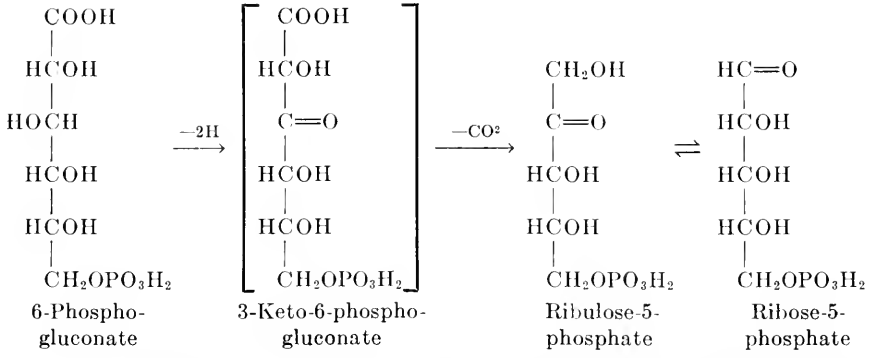

FIG. 2. Formation of pentose phosphates from 6-phosphogluconate according to Horecker *et al.*[16]

librium is reached when 70 to 80 % of the pentose phosphate is in the form of ribose-5-phosphate, this interconversion being catalyzed by a pentose phosphate isomerase. Similar results were obtained with a partially purified liver 6-phosphogluconate dehydrogenase preparation.[18]

The occurrence of ribulose-5-phosphate as a precursor of ribose-5-phosphate suggested that 6-phosphogluconate might be oxidized at the 3-position to give the hypothetical intermediate 3-keto-6-phosphogluconate, which on decarboxylation would yield ribulose-5-phosphate according to Fig. 2.[16] No arabinose-5-phosphate was formed with this purified dehydrogenase preparation, indicating that this is not an intermediate in the conversion of 6-phosphogluconate to ribose-5-phosphate.

Axelrod and Jang[19] have recently obtained similar results with a partially purified spinach leaf preparation, using C^{14}-labeled 6-phosphogluconate.

3. Metabolism of Ribulose-5-Phosphate

a. Formation of Triose Phosphate

Triose phosphate has been identified as one of the products of pentose phosphate cleavage by enzymes of red cells,[20] bacteria and yeast,[21] liver,[22,23]

[18] J. E. Seegmiller and B. L. Horecker, *J. Biol. Chem.* **194**, 261 (1952).

[19] B. Axelrod, R. S. Bandurski, C. M. Greiner, and R. Jang, *J. Biol. Chem.* **202**, 619 (1953).

[20] Z. Dische, *Naturwissenschaften* **26**, 252 (1938).

[21] E. Racker, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 1, p. 147. Johns Hopkins Press, Baltimore, 1951; H. Z. Sable, *Federation Proc.* **10**, 241 (1951).

[22] B. L. Horecker and P. Z. Smyrniotis, *J. Am. Chem. Soc.* **74**, 2123 (1952).

[23] G. E. Glock, *Biochem. J.* **52**, 575 (1952).

and leaves.[19] The C_2 fragment, which is not free glycolaldehyde,[19,23-25] has not, however, been characterized. It may be glycolaldehyde in a "bound"[26] or "active"[27] form which can also apparently arise enzymically from dihydroxymaleic acid or hydroxypyruvic acid.[28] Evidence has been obtained with both yeast[25] and liver[29] enzymes that ribulose-5-phosphate and not ribose-5-phosphate is the true substrate for the pentose phosphate-splitting enzyme, since crude enzyme preparations attack both substrates readily whereas partially purified preparations, although still containing some pentose phosphate isomerase, attack ribulose-5-phosphate much more readily than the ribose ester. Racker *et al.*[27] have recently isolated a crystalline enzyme, free from pentose phosphate isomerase, from baker's yeast which catalyzes the cleavage of ribulose-5-phosphate with the formation of D-glyceraldehyde-3-phosphate, which was identified. This cleavage only occurs on the addition of an "acceptor aldehyde" such as ribose-5-phosphate or glycolaldehyde.

b. Synthesis of Sedoheptulose-7-phosphate and Hexosemonophosphate

Degradation of pentose phosphate by blood hæmolysates,[20,24,30] partially purified enzyme preparations from liver[23,26,31] and bone marrow,[18] and by many other mammalian tissues and tumors[32] is accompanied by the formation of hexosemonophosphate. This synthesis of hexosemonophosphate has been shown to proceed without passing through the intermediate stage of fructose diphosphate.[30,33] Dische[24] and Glock,[23] using, respectively, blood hæmolysates and partially purified liver preparations, obtained approximately 75 % conversion of the ribose-5-phosphate that was degraded into hexosemonophosphate. This is considerably in excess of the hexosemonophosphate which could be derived solely from the triose fragment of the pentose phosphate. Evidence was also obtained that fructose monophosphate (presumably fructose-6-phosphate) was formed first and then gradually converted into glucose-6-phosphate by the action of hexose phosphate isomerase. Sedoheptulose monophosphate was soon detected as an inter-

[24] Z. Dische, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 1, p. 171. Johns Hopkins Press, Baltimore, 1951.
[25] G. De la Haba and E. Racker, *Federation Proc.* **11**, 201, (1952).
[26] B. L. Horecker, *J. Cellular Comp. Physiol.* **41**, Suppl.1, 137 (1953).
[27] E. Racker, G. De la Haba, and I. G. Leder, *J. Am. Chem. Soc.* **75**, 1010 (1953).
[28] S. Akabori, K. Uehara, and I. Miramatsu, *Proc. Japan Acad.* **28**, 39 (1952).
[29] B. L. Horecker and P. Z. Smyrniotis, *Federation Proc.* **11**, 232 (1952).
[30] Z. Dische, *Abstr. 1st Intern. Congr. Biochem., Cambridge* p. 572 (1949).
[31] M. J. Waldvogel and F. Schlenk, *Arch. Biochem.* **14**, 484 (1947); **22**, 185 (1949).
[32] Gertrude E. Glock and P. McLean, *Biochem. J.* **56**, 171 (1954).
[33] F. Dickens and Gertrude E. Glock, *Biochem. J.* **50**, 81 (1951).

mediate product in the formation of hexosemonophosphate from pentose phosphate,[19,34,35] following the initial discovery of Benson *et al.*[36] that this is one of the first phosphorylated products to be formed in photosynthesis. Using a purified pentose phosphate-splitting enzyme from liver together with crystalline muscle aldolase to catalyze the condensation of the cleavage products, Horecker and Smyrniotis[22] obtained approximately one mole of sedoheptulose phosphate from every two moles of pentose phosphate that disappeared. Ketoheptose was detected by paper chromatography[37] and by the characteristic absorption band at 600 mμ in the Bial reaction and was characterized by the preparation of sedoheptulosan tetrabenzoate.[38] The rate of acid hydrolysis suggested that it was sedoheptulose-7-phosphate. A similar conversion of pentose phosphate into sedoheptulose-7-phosphate was also found with a spinach enzyme.[39] This enzyme was found to contain firmly bound thiamine pyrophosphate which was shown to be essential for the synthesis of sedoheptulose-7-phosphate. The same enzyme preparation catalyzed the condensation of L-erythrulose with D-glyceraldehyde-3-phosphate to form a mixture of pentose phosphate and heptulose phosphate, and the following sequence of reactions was suggested to account for the formation of hexosemonophosphate from pentose phosphate:[40]

(1) D-ribulose-5-P $\rightleftharpoons$ 2 D-glyceraldehyde-3-P + L-erythrulose
(2) L-erythrulose + D-glyceraldehyde-3-P $\rightleftharpoons$ sedoheptulose-7-P
(3) sedoheptulose-7-P + D-glyceraldehyde-3-P $\rightarrow$ fructose-6-P + tetrose P

Reaction (3) is catalyzed by enzymes from brewer's yeast and liver and the reaction mechanism has been clarified by the use of C^{14}-labeled triose phosphate.[41] The dihydroxyacetone group is transferred from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate with the formation of fructose-6-phosphate and tetrose phosphate (presumably erythrose-4-phosphate), as shown in Fig. 3. The enzyme catalyzing this reaction has been called "transaldolase" since it promotes the transfer of aldol linkages rather than their hydrolytic cleavage. The fate of the residual tetrose phosphate has not been satisfactorily explained.

[34] B. L. Horecker, *Abstr. 2nd Intern. Congr. Biochem., Paris* p. 292 (1952).
[35] Z. Dische and E. Pollaczek, *Abstr. 2nd Intern. Congr. Biochem,. Paris* p. 289 (1952).
[36] A. A. Benson, J. A. Bassham, and M. Calvin. *J. Am. Chem. Soc.* **73,** 2970 (1951).
[37] R. Kleystrand and A. Nordal, *Acta Chem. Scand.* **4,** 1320 (1950).
[38] W. T. Haskins, R. M. Hann, and C. S. Hudson, *J. Am. Chem. Soc.* **74,** 2198 (1952).
[39] B. L. Horecker and P. Z. Smyrniotis, *J. Am. Chem. Soc.* **75,** 1009 (1953).
[40] B. L. Horecker, P. Z. Smyrniotis, and H. Klenow, *Federation Proc.* **12,** 219 (1953).
[41] B. L. Horecker and P. Z. Smyrniotis, *J. Am. Chem. Soc.* **75,** 2021 (1953).

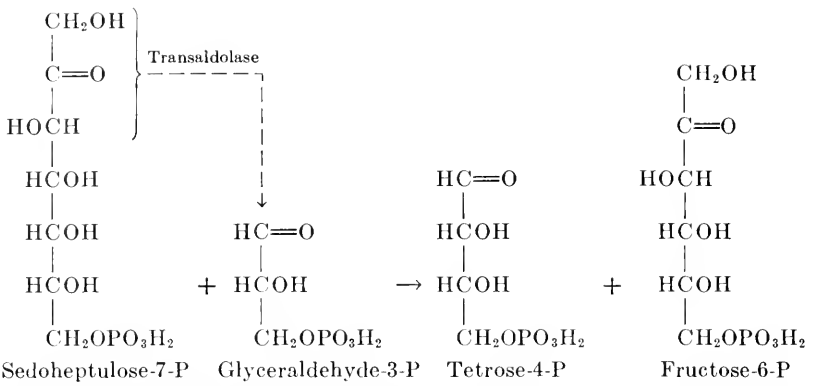

FIG. 3. Method of formation of fructose-6-phosphate from sedoheptulose-7-phosphate according to Horecker and Smyrniotis.[41]

III. Pentose Phosphate Formation in Photosynthesis

Calvin and Benson[42] and their co-workers[43] have shown that after very short periods of photosynthesis in $C^{14}O_2$, phosphoglyceric acid contains most of the radioactivity, this being located almost exclusively in the carboxyl group. Numerous phosphorylated products follow the initial formation of phosphoglyceric acid and these have been studied in green algæ as well as in preparations from leaves of various higher plants, using C^{14} and P^{32} as tracers. The identification of small quantities of radioactive compounds has been accomplished by paper chromatography with non-radioactive carriers, coincidence between the radioautograph and the spot produced by the color reaction of a particular carrier being taken as strong evidence for their identity. Sedoheptulose monophosphate, ribose and ribulose monophosphates, and ribulose-1,5-diphosphate were among the products detected during the first few seconds of photosynthesis,[36,44,45] most of the pentose phosphate being in the form of ribulose diphosphate. The extreme rapidity with which sedoheptulose phosphate, in particular, is labeled is emphasized by the figures given by Buchanan *et al.*[46] After a period of photosynthesis as short as ten seconds by a soybean leaf prepara-

[42] M. Calvin and A. A. Benson, *Science* **109**, 140 (1949).

[43] A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka, *J. Am. Chem. Soc.* **72**, 1710 (1950).

[44] A. A. Benson, *J. Am. Chem. Soc.* **73** 2971 (1951).

[45] A. A. Benson, J. A. Bassham, M. Calvin, A. G. Hall, H. E. Hirsch, S. Kawaguchi, V. H. Lynch, and N. E. Tolbert, *J. Biol. Chem.* **196**, 703 (1952).

[46] J. G. Buchanan, J. A. Bassham, A. A. Benson, D. F. Bradley, M. Calvin, L. L. Daus, M. Goodman, P. M. Hayes, V. H. Lynch, L. T. Norris, and A. T. Wilson, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 440. Johns Hopkins Press, Baltimore, 1952.

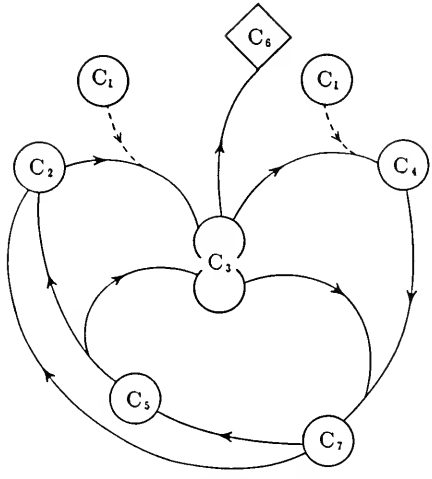

FIG. 4. The cyclic conversion of phosphate esters in photosynthesis (after Calvin and co-workers[46]).

tion, phosphoglyceric acid, sedoheptulose monophosphate, fructose monophosphate, and pentose mono- and diphosphates contained, respectively, 32%, 24%, 19%, and 9% of the total radioactivity (C^{14}). More recent kinetic studies on steady-state photosynthesis[47] show that the steady-state concentration of ribulose diphosphate is relatively high, the concentrations of phosphoglyceric acid, ribulose diphosphate, sedoheptulose phosphate, fructose phosphate, and glucose phosphate being, respectively, 1.4, 0.5, 0.18, 0.12, and 0.4 μM. per ml. *Scenedesmus* cells. Although degradation data are not yet available, ribulose diphosphate is believed to arise from sedoheptulose phosphate, and the latter to be formed by aldolase condensation of triose phosphate and a tetrose (presumably erythrose). It has been suggested[34,45] that sedoheptulose phosphate and ribulose diphosphate are involved in the regeneration of the C_2 fragments utilized in CO_2 fixation rather than in the synthesis of hexose. This is supported by Calvin and Massini's findings[47] that the sudden rise in phosphoglyceric acid during a period of darkness, following a preliminary period of illumination, is accompanied by a decrease in both ribulose diphosphate and sedoheptulose phosphate. Since the rate of production of phosphoglyceric acid was higer during the first minute of darkness than in photosynthesis, it was tentatively suggested that the C_3 cleavage product of ribulose diphosphate is triose phosphate during photosynthesis and phosphoglyceric acid in the dark, this hypothesis being supported by the fact that triose phosphate also decreases in the dark. Experiments with iodoacetamide-poisoned

[47] M. Calvin and P. Massini, *Experientia* **8**, 445 (1952).

Chlorella cells showed that there was practically no incorporation of C^{14} into ribulose diphosphate and much less into phosphoglyceric acid than in the control cells. This was taken as an indication that the cleavage of heptose and pentose phosphates might be dependent on sulfhydryl enzymes (see also Sect. IV.2). A scheme for the cyclic conversion of phosphate esters in photosynthesis is shown in Fig. 4.

Although Calvin and his co-workers consider hexose phosphates to be synthesized exclusively from triose phosphate by a reversal of the glycolytic route, recent work of Horecker[26] indicates that the direct oxidative pathway may serve as an additional source of hexose in photosynthesis. He has shown that when energy is supplied in the form of reduced TPN, pentose phosphate and CO_2 can be reduced to glucose-6-phosphate. The recent important observations of Vishniac and Ochoa[48] indicate that photosynthetic mechanisms may be able to provide the reduced coenzyme for these reactions.

IV. Significance of the Hexosemonophosphate Oxidative Pathway

1. Distribution

The enzymes of this oxidative pathway of carbohydrate metabolism are widely distributed throughout the animal and vegetable kingdom and have been demonstrated in a variety of mammalian tissues and tumors,[32,33] in lower animals,[49] in higher plants and algae,[50,51] and in yeast and many bacteria.[52,53]

Recent quantitative results of Glock and McLean[32] for glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities of some normal mammalian tissues are shown in Table I. The most interesting results are the very high levels of activity of both dehydrogenases in adrenal cortex and lactating mammary gland and the strikingly low levels in muscle. The physiological significance of these findings is, however, at present obscure. If one of the main functions of this pathway is to supply ribose-5-phosphate for incorporating into ribonucleic acid, it would be expected to play an important part in the metabolism of tumors and other rapidly dividing cells. The levels of activity of both dehydrogenases in a variety of experimental and spontaneous tumors were, however, found to fall within the limits of activity of normal tissues. This method of approach is, however,

[48] W. Vishniac and S. Ochoa, *J. Biol. Chem.* **195**, 75 (1952).
[49] S. S. Cohen, *Biol. Bull.* **99**, 369 (1950); **101**, 237 (1951).
[50] G. E. Glock and P. McLean, *Biochem. J.* **55**, 440 (1953).
[51] P. K. Stumpf *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 46. Johns Hopkins Press, Baltimore, 1952.
[52] W. A. Wood and R. F. Schwerdt, *J. Cellular Comp. Physiol.* **41**, Suppl. 1, 165 (1953); *J. Biol. Chem.* **201**, 501 (1953).
[53] R. D. DeMoss, *J. Cellular Comp. Physiol.* **41**, Suppl. 1, 207 (1953).

TABLE I

LEVELS OF GLUCOSE-6-PHOSPHATE AND 6-PHOSPHOGLUCONATE DEHYDROGEANSE ACTIVITIES IN ADULT MAMMALIAN TISSUES

Tissue	Enzyme activity in units/g. tissue[a]		
	G-6-P dehydrogenase (pH 7.6)	6-PG dehydrogenase (pH 9.0)	6-PG dehydrogenase (pH 7.6)
Adrenal gland[6]	163 ± 25	350 ± 25	185 ± 9
Adrenal cortex[2b]	730	315	232
Adrenal medulla[2b]	67	114	67
Spleen[4]	305 ± 31	96 ± 7	64 ± 3
Thymus[6]	98 ± 1	87 ± 9	49 ± 5
Liver (Female)[12]	104 ± 12	290 ± 16	130 ± 12
Liver (Male)[18]	46 ± 3	147 ± 10	59 ± 8
Lung[5]	88 ± 6	106 ± 7	59 ± 3
Kidney[3]	69 ± 7	62 ± 5	56 ± 3
Brain[6]	32 ± 3	22 ± 1	11 ± 1
Cardiac muscle[6]	26 ± 13	34 ± 7	18 ± 3
Skeletal muscle[6]	8 ± 1	15 ± 0.5	8 ± 0.3
Mammary gland			
20th day of pregnancy	86	92	50
10th day of lactation	1,728	673	409
21st day of lactation	5,452	1,734	883
2nd day of involution	50	93	45

[a] A unit of enzyme activity is defined as the quantity of enzyme which reduces 0.01 μM. TPN/min. at 20°
[b] Ox tissues (rat tissues otherwise used).

somewhat limited since only the maximum enzymic capacities are being measured and these do not necessarily operate in the intact cell where other regulatory factors probably limit or control the activity of this pathway.

Both glucose-6-phosphate and 6-phosphogluconate dehydrogenases are located exclusively in the soluble fraction (cell sap) of tissue homogenates.[50,54] It is of interest that the glycolytic enzymes have the same intracellular distribution.[55]

2. As an Alternative Pathway to Glycolysis

Before the existence of the direct oxidative pathway was established with certainty in animal tissues, considerable indirect evidence had accumulated indicating that although glycolysis is the principal mechanism of carbohydrate breakdown under anærobic conditions, other mechanisms

[54] Gertrude E. Glock and P. McLean, *Nature* **170,** 119 (1952).
[55] G. A. LePage and W. C. Schneider, *J. Biol. Chem.* **176,** 1021 (1948).

can operate ærobically. Thus several workers have shown,[56] using both intact muscles and tissue slices, that, after glycolysis is inhibited by iodoacetate, oxidation of carbohydrate can still continue at a normal or only slightly diminished rate.

Oxidation of carbohydrate by the direct oxidative pathway proceeds independently of glycolysis since the primary oxidations of both glucose-6-phosphate and 6-phosphogluconate are in general TPN-specific (cf. some bacteria),[52,53] show no requirement for either inorganic phosphate or ATP, and are almost unaffected by concentrations of iodoacetate and fluoride which completely inhibit glycolysis.[33] Although this pathway is relatively resistant to iodoacetate, liver 6-phosphogluconate dehydrogenase has been shown to be a sulfhydryl enzyme[50] and it is highly probable that this is also true of glucose-6-phosphate dehydrogenase[50] and pentose phosphate isomerase.[57] The formation of sedoheptulose-7-phosphate from pentose-5-phosphate by spinach leaf preparations is also inhibited by sulfhydryl-combining compounds.[19]

The cyclic nature of the direct oxidative pathway has already been emphasized. The primary oxidation product of glucose-6-phosphate has been shown by Cori and Lipmann[58] to be 6-phospho-δ-gluconolactone, the subsequent hydrolysis to 6-phosphogluconate probably being spontaneous. Oxidative decarboxylation of 6-phosphogluconate gives rise to ribulose-5-phosphate (in equilibrium with ribose-5-phosphate), which is then degraded into triose phosphate and a C_2 fragment, and glucose-6-phosphate eventually resynthesized with the intermediate formation of sedoheptulose-7-phosphate and fructose-6-phosphate. An important new development has been the demonstration by Horecker that both formation of 6-phosphogluconolactone from glucose-6-phosphate[26] and of ribulose-5-phosphate from 6-phosphogluconate[59] are reversible. Experiments of Cohen, however, in which he determined the utilization of C_1-labeled gluconate by adapted intact cells of *E. coli*, showed that the formation of 6-phosphogluconate from glucose-6-phosphate is practically irreversible in this organism.[60]

The main reaction of these two alternative pathways of carbohydrate metabolism, namely by the anærobic glycolytic route and the direct oxidative pathway are presented in Fig. 5, which also serves to emphasize both their common origin in glucose-6-phosphate and their convergence at triose phosphate. The relative importance of these two pathways under

[56] E. Shorr, *Cold Spring Harbor Symposia Quant. Biol.* **7**, 323 (1939); E. Stotz, *Advances in Enzymol.* **5**, 129 (1945).

[57] B. Axelrod and R. Jang, *Federation Proc.* **12**, 172 (1953).

[58] O. Cori and F. Lipmann, *J. Biol. Chem.* **194**, 417 (1952).

[59] B. L. Horecker and P. Z. Smyrniotis, *J. Biol. Chem.* **196**, 135 (1952).

[60] S. S. Cohen, *Nature* **168**, 746 (1951); *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 1, p. 148. Johns Hopkins Press, Baltimore, 1951.

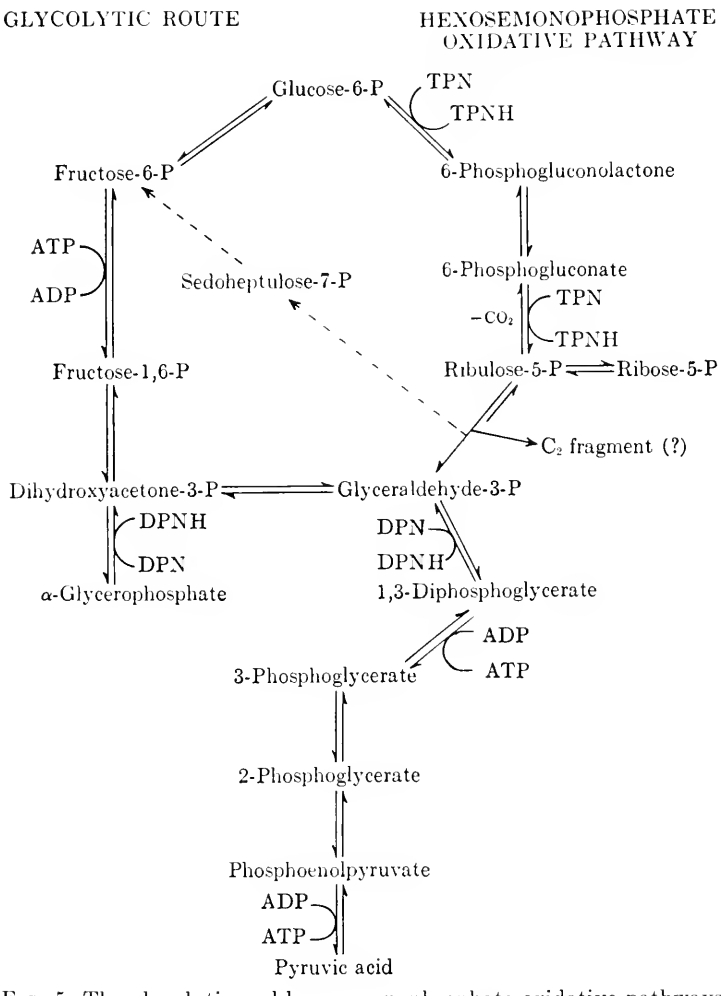

FIG. 5. The glycolytic and hexosemonophosphate oxidative pathways.

different physiological conditions is still, however, chiefly a matter of conjecture. Englehardt and Sakov[61] suggested that the redox potential would determine the route of carbohydrate metabolism, since phosphohexose kinase was found to be very sensitive to O_2 and various redox dyes. In the living cell, a multiplicity of factors presumably regulate these metabolic pathways including probably electrolyte distribution, O_2 tension, and the influence of hormones. A few attempts have, however, been made to assess

[61] W. A. Englehardt and N. E. Sakov, *Biokhimiya* **8**, 9 (1943), quoted by F. Dickens *in* "The Enzymes" (Sumner and Myrbäck, eds.), Vol. 2, p. 624. Academic Press, New York.

TABLE II

C_1 Recovery in CO_2 Produced During Glucose Utilization by E. coli

Physiological condition	Moles CO_2/mol. glucose	$BaCO_3$,[a] cts./min./mg. C	Recovery[b] C_1 in CO_2, per cent	Recovery[c] C_1 (theory), per cent	Excess per cent
Oxidation without growth	3.22	212	54.4	53.7	0.7
Growth	1.32	156	37.7	22.0	15.7
T_2 synthesis	1.38	121	29.1	23.0	6.1

[a] After known dilution of CO_2 with carbonate.

[b] Calculated from the following equation:

$$\% \text{ recovery} = \frac{BaCO_3 \text{ isolated} \times \text{specific activity } (BaCO_3)/\text{mg. C} \times 100}{\mu\text{M. glucose} \times \text{mol. wt. } BaCO_3 \times \text{specific activity (glucose-C}_1)}$$

[c] Calculated from maximal liberation of isotope from CH_3-labeled pyruvate

$$\% \ C_1 \text{ in } CO_2 = \frac{\text{moles } CO_2 \text{ liberated}}{6} \times 100$$

the relative importance of these alternative pathways. The most notable contribution has been made by Cohen,[60] who studied the metabolism of glucose-1-C^{14} by intact cells of *E. coli*. Whereas utilization of glucose by the direct oxidative pathway would result in preferential conversion of C_1 to CO_2, this would not occur if the glycolytic scheme were operating, since most of the CO_2 produced during anærobic glycolysis arises from the carboxyl group of pyruvic acid which is derived from C_3 or C_4 of the original glucose. The results obtained by Cohen are given in Table II. When glucose was oxidized by resting bacteria there was no indication of preferential liberation of CO_2 from C_1 of glucose. Under conditions of growth, however, an average of 37 % of the C_1 was recovered in the liberated CO_2, which is significantly in excess of the value of 23 % which would be obtained if the glucose were converted to pyruvate anærobically and then metabolized completely. The minimum value for glucose degradation by the oxidative pathway would then be this excess of 14 % and the maximum value would be 37 %. This figure agrees well with the results of later experiments[62] in which it was shown that extracts of growing *E. coli* cells contained sufficient glucose-6-phosphate and 6-phosphogluconate dehydrogenases to account for approximately 40 % of the total metabolism of carbohydrate. When *E. coli* was infected with T_2r^+ bacteriophage, growth was suspended and activity confined exclusively to the synthesis of virus components.[10] Under these conditions, the excess of C_1 in the liberated CO_2 was markedly de-

[62] D. B. M. Scott and S. S. Cohen, *Federation Proc.* **11**, 284 (1952); *Biochem. J.* **55**, 33 (1953).

creased.[60] This may be interpreted as a diversion of glucose utilization from the oxidative pathway to the glycolytic route and occurred under conditions in which ribonucleic acid synthesis was eliminated and deoxyribonucleic acid synthesis stimulated.

Also of interest are the recent investigations of Gibbs, Gunsalus, and DeMoss on the fermentation of glucose by *Leuconostoc mesenteroides*[63,64] and *Pseudomonas lindneri*[65] to CO_2, ethanol, and lactic acid. Isotope data have indicated the participation of an anærobic hexose monophosphate pathway in both organisms since, when glucose-1-C^{14} was used, all the radioactivity was recovered in the CO_2 originating from C_1 of the glucose whereas if the fermentation had proceeded by the anærobic glycolytic, route, C_1 of the glucose would have appeared in the ethanol.[66] *L. mesenteroides* was shown to possess an active glucose-6-phosphate dehydrogenase which is active with either DPN or TPN in contrast to the TPN-specificity of this enzyme from other sources.

The utilization of glucose-1-C^{14} by growing yeast has been investigated by Gilvarg,[67] but, since no evidence was found of preferential conversion of C_1 to CO_2, it was concluded that the direct oxidative pathway is not a major route of carbohydrate degradation in growing yeast.

3. As a Source of Pentose Phosphate for Nucleic Acid Synthesis

The experiments of Cohen[60] on the utilization of glucose by intact cells of *E. coli* both during growth, when approximately three times as much PNA as DNA is being synthesized,[10] and during virus infection, when DNA only is formed, were described in the previous section. Utilization of C_1-labeled gluconate was also studied. Gluconate-adapted *E. coli* produces a specific gluconokinase[68] catalyzing the phosphorylation of gluconate to 6-phosphogluconate, which then enters the direct oxidative pathway. Since growth on gluconate was almost as good as on glucose, and 6-phosphogluconate cannot be converted enzymically to glucose-6-phosphate in this organism, it appears that uninfected *E. coli* cells can produce sufficient ribose and deoxyribose for their needs via the direct oxidative pathway. During virus infection however, when the demand for deoxyribose is greatly increased, gluconate is much inferior to glucose for virus and DNA synthesis. Cohen concluded that ribose for PNA synthesis possibly originates in the direct oxidative pathway but that deoxyribose is generated more readily from the anærobic glycolytic route.

[63] M. Gibbs and R. D. DeMoss, *Federation Proc.* **10,** 189 (1951).
[64] I. C. Gunsalus and M. Gibbs, *J. Biol. Chem.* **194,** 871 (1952).
[65] M. Gibbs and R. D. DeMoss, *Arch. Biochem. and Biophys.* **34,** 478 (1951).
[66] D. E. Koshland and F. H. Westheimer, *J. Am. Chem. Soc.* **72,** 3383 (1950).
[67] C. Gilvarg, *J. Biol. Chem.* **199,** 57 (1952).
[68] S. S. Cohen and D. B. M. Scott, *Nature* **166,** 781 (1950).

Significant incorporation of labeled P into nucleic acids of *E. coli* growing in the presence of fructose-6-P^{32} has been reported by Roberts and Wolffe.[69] Since far less effect was obtained with glucose-6-P^{32} and none with fructose-1,6-P^{32} they suggested that fructose-6-phosphate does not pass through glucose-6-phosphate as an intermediate but is oxidized by a similar independent process giving rise to pentose phosphate and nucleic acid. This interesting observation requires confirmation. Incorporation of isotope into nucleic acid was also found on feeding gluconate uniformly labeled with C^{14} to rats,[70] but no degradation was carried out to determine to what extent the pentose was labeled.

Bernstein[71] has studied the *in vivo* synthesis of ribose in the chicken after feeding acetate-1-C^{14}. The patterns of incorporation of the isotope into glucose (glycogen) and PNA ribose differed to such an extent that the direct conversion of hexose to ribose was excluded as a major pathway for ribose synthesis. The results could, however, be explained by a condensation of C_2 and C_3 units.

It is obvious that far more experimental data are required before it will be possible to evaluate precisely the part played by the direct oxidative pathway in the synthesis of PNA. Such investigations are, however, restricted by the impermeability of most cells to phosphorylated sugars.

V. Other Methods of Biosynthesis of Pentose Phosphates

1. Condensation of C_2 and C_3 Units

a. Synthesis of Xyloketose-1-phosphate

The aldolase-catalyzed condensation of dihydroxyacetone phosphate and glycolaldehyde to form ketopentose-1-phosphate was originally reported by Lohmann.[72] It has subsequently been prepared by Racker[73] with crystalline muscle aldolase and generally assumed to be xyloketose-1-phosphate. The same ketopentose-1-phosphate was presumably also synthesized from glycolaldehyde and triose phosphate by extracts of *Micrococcus pyogenes*.[74] The final characterization from the rate of acid hydrolysis[23] and by identification of the sugar as D-xylulose[75] is, however, relatively recent. This pentose phosphate is of doubtful metabolic significance. Since only *trans*-linkages are formed from aldolase-catalyzed condensations, the direct formation of ribose-5-phosphate by this mechanism is precluded.

[69] I. Z. Roberts and E. L. Wolffe, *Arch. Biochem. and Biophys.* **33,** 165 (1951).
[70] M. R. Stetten and DeW. Stetten, Jr., *J. Biol. Chem.* **187,** 241 (1950).
[71] I. A. Bernstein, *J. Am. Chem. Soc.* **73,** 5003 (1951).
[72] K. Lohmann, *Angew. Chem.* **49,** 327 (1936).
[73] E. Racker, *Federation Proc.* **7,** 180 (1948).
[74] J. Marmur and F. Schlenk, *Arch. Biochem. and Biophys.* **31,** 154 (1951).
[75] L. Hough and J. K. N. Jones, *J. Chem. Soc.* **1952,** 4047; R. S. Forrest, L. Hough, and J. K. N. Jones, *Chemistry & Industry* **1093** (1951).

b. Synthesis of Deoxyribose-5-phosphate

Racker[76] has shown that extracts of *E. coli* catalyze the following reversible reaction:

$$\text{glyceraldehyde-3-P} + \text{acetaldehyde} \rightleftharpoons \text{deoxyribose-5-P}$$

Deoxyribose phosphate aldolase (DR aldolase), which catalyzes this reversible aldol condensation, is widely distributed in microorganisms and animal tissues, thymus and liver being particularly rich in this enzyme. It has been purified from *E. coli* extracts and found to be unaffected by NaF (10^{-2} *M*) and iodoacetate (10^{-4} *M*) but inhibited by octyl alcohol, chloral hydrate, and propionaldehyde. Enzymically formed deoxyribose-5-phosphate has been isolated and characterized and found to be utilized by crude bacterial extracts for the formation of nucleosides. Deoxyribose-5-phosphate can also be formed from ribose-5-phosphate and more readily from ribulose-5-phosphate in the presence of acetaldehyde and partially purified enzymes from baker's yeast and *E. coli*. The yeast enzyme catalyzes the formation of glyceraldehyde-3-phosphate from ribulose-5-phosphate and the *E. coli* enzyme the subsequent condensation with acetaldehyde.

Since DR aldolase has a very low affinity for acetaldehyde, this may not be the natural substrate for this enzyme. Racker suggests that in the cell an aldehyde linked to a purine or pyrimidine precursor may normally condense with glyceraldehyde-3-phosphate, thus accomplishing a direct synthesis of deoxyribose nucleotide. Hammarsten *et al.*[77] have suggested that deoxyribose may arise from ribose while the latter is in glycosidic linkage.

c. Synthesis of Ribulose-5-phosphate

Akabori *et al.*[28] demonstrated the formation of a ribose phosphate, believed to be ribose-5-phosphate, on incubating fructose-1,6-diphosphate and dihydroxymaleic acid with minced rabbit muscle. It was suggested that carboligase catalyzed the condensation of glyceraldehyde-3-phosphate and hydroxypyruvic acid (formed by decarboxylation of dihydroxymaleic acid) with elimination of CO_2 and formation of ribulose-5-phosphate, in equilibrium with ribose-5-phosphate, according to Fig. 6. Dihydroxymaleic acid could not be replaced by glycolaldehyde.

This somewhat novel method of synthesis of pentose phosphate was confirmed by Racker *et al.*[27] Using a crystalline enzyme from baker's yeast, which catalyzes the cleavage of ribulose-5-phosphate, they obtained decarboxylation of hydroxypyruvic acid in the presence of an "acceptor aldehyde." With D- or DL-glyceraldehyde-3-phosphate as acceptor, decar-

[76] E. Racker, *Nature* **167**, 408 (1951); *J. Biol. Chem.* **196**, 347 (1952).
[77] E. Hammarsten, P. Reichard, and E. Saluste, *J. Biol. Chem.* **183**, 105 (1950).

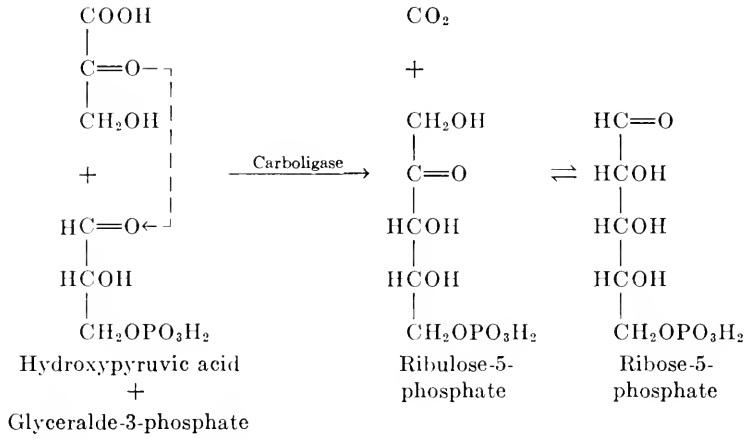

FIG. 6. Carboligase-catalyzed formation of pentose phosphate according to Akabori *et al.*[28]

boxylation of hydroxypyruvic acid resulted in the formation of ribulose-5-phosphate. Since this formation of ribulose-5-phosphate represents a ketol condensation and no free glycolaldehyde is formed, Racker assumes the formation of "active" glycolaldehyde which condenses with the "acceptor aldehyde" to form a keto-sugar. This enzyme has been called "transketolase." Thiamine pyrophosphate is necessary for its activity. This is also true of the liver and spinach leaf enzymes.[127]

2. Decarboxylation of Uronic Acids

The first indication that pentose might be derived enzymically from uronic acid was the isolation of D-xylose from a decaying mince incubated with D-glucuronic acid.[78] It was subsequently observed in numerous plant gums and mucilages that when a single uronic acid and a single pentose are present together, the two are frequently in a homologous series, for example D-glucuronic acid and D-xylose, and D-galacturonic acid and L-arabinose. These findings led to the hypothesis that the uronic acids are directly decarboxylated to the homologous pentoses.[79] However, the isolation of D-mannuronic acid and the failure to detect D-lyxose in plant products, and the widespread occurrence of D-ribose but absence of D-alluronic acid has thrown some doubt on the validity of this hypothesis. This problem is discussed in detail by Hirst.[80]

Cohen decided to test this hypothesis experimentally using uronic acid-

[78] E. Salkowski and C. Neuberg, *Z. physiol. Chem.* **36,** 261, (1902).
[79] J. M. Gulland, *J. Chem. Soc.* **1944,** 208; E. L. Hirst, *J. Chem. Soc.* **1942,** 70.
[80] E. L. Hirst, *J. Chem. Soc.* **1949,** 522.

adapted *E. coli*.[81] Cells adapted to either glucuronic acid or galacturonic acid could oxidize or ferment both uronic acids but not D-xylose, L-arabinose, or D-ribose. It was concluded that, at least in the K 12 strain of *E. coli*, the metabolism of uronic acid does not proceed by the direct decarboxylation of uronic acid to pentose. An alternative method of uronic acid metabolism was suggested, namely, phosphorylation of glucuronic acid followed by decarboxylation to D-xylose phosphate, which might be the intermediate in pentose formation. The findings of Heald[82] that D-glucuronate and D-xylose gave similar fermentation products with rumen bacteria from the sheep may, perhaps, be taken as indirect evidence for the formation of D-xylose by decarboxylation of D-glucuronic acid.

The only other experimental evidence is that of Enklewitz and Lasker,[83] who reported that whereas administration of D-glucuronic acid to normal subjects produced no pentosuria, there was a marked increase in the excretion of L-xyloketose in pentosurics. These results, however, are not very convincing and require confirmation preferably using labeled glucuronic acid.

3. In Vivo Synthesis from Noncarbohydrate Sources

Evidence is gradually accumulating to indicate extensive formation of PNA ribose from sources other than carbohydrate. Using C^{14}-labeled substrates, it has been shown that significant amounts of PNA ribose can be formed in the chicken from glycine,[84] acetate,[71] and formate.[85] When doubly labeled acetate (C^{13} in the methyl group and C^{14} in the carboxyl group) was fed to rats,[86] there was preferential incorporation of the α-C indicating that acetate is probably not an immediate precursor of ribose but enters it by some indirect route.

4. Miscellaneous

In the preliminary experiments of Charalampous[87] on the incorporation of formaldehyde into phosphorylated sugars, he found that a liver enzyme, distinct from aldolase, catalyzed the condensation of formaldehyde with triose phosphate. The reaction products were separated by ion-exchange chromatography and, although the main component was a tetrose phosphate (later identified as erythrulose-1-P),[88] approximately 10% was a

[81] S. S. Cohen, *J. Biol. Chem.* **177,** 607 (1949).
[82] P. J. Heald, *Biochem. J.* **50,** 503 (1952).
[83] M. Enklewitz and M. Lasker, *J. Biol. Chem.* **110,** 443 (1935).
[84] B. Löw, *Acta Chem. Scand.* **4,** 294 (1950).
[85] I. A. Bernstein, *Federation Proc.* **11,** 187 (1952).
[86] B. Löw, *Acta Chem. Scand.* **6,** 304 (1952).
[87] F. Charalampous, *Federation Proc.* **11,** 196 (1952).
[88] F. C. Charalampous and G. C. Mueller, *J. Biol. Chem.* **201,** 161 (1953).

ribose ester and 7 % an ester of either L-arabinose or glucose. This method of formation of pentose phosphate does not appear to have been investigated further.

Horecker and Smyrniotis[41] have suggested that the transfer of a dihydroxyacetone group by "transaldolase" from one phosphorylated sugar to another, which they demonstrated in connection with the formation of fructose-6-phosphate from sedoheptulose-7-phosphate (see Sect. II.3.*b.*), is probably not confined to this particular reaction. It may be a general method of synthesis of 2-keto-sugar phosphates including 2-ketopentose phosphates.

Although not completely relevant in connection with the synthesis of pentose phosphates, the formation of ketopentoses by oxidation of pentitols should be mentioned. This has been demonstrated by Hudson and his co-workers,[89] who found that *Acetobacter suboxydans* produced good yields of D-xylulose from D-arabitol.

VI. Interconversion of Pentose Phosphates and Pentoses

1. Phosphopentose Isomerase

The presence of a specific phosphoribose isomerase catalyzing the interconversion of ribulose-5-phosphate and ribose-5-phosphate was first demonstrated in a purified yeast enzyme preparation by Horecker and Smyrniotis.[15,16] At equilibrium, approximately 80 % of the ribose ester is present. The ubiquitous distribution of these two pentose phosphates suggests that this also applies to the isomerase. Purified phosphoribose isomerase from alfalfa leaves was found to be inhibited by *p*-chloromercuribenzoate.[57]

2. Phosphopentomutases

a. Phosphoribomutase

The existence of an enzyme catalyzing the formation of ribose-5-phosphate from ribose-1-phosphate was suggested by Schlenk.[90] Kalckar[91] showed that the transformation of inosine into hypoxanthine by crude liver nucleoside phosphorylase in the presence of inorganic phosphate was accompanied by the formation of ribose-1-phosphate. This was converted into an acid-stable ester, presumably ribose-5-phosphate, by the presence of phosphoribomutase in the liver extract. Later work confirmed this[92,93] and, on account of the rapid surface denaturation of this mutase on foam-

[89] R. M. Hann, E. B. Tilden, and C. S. Hudson, *J. Am. Chem. Soc.* **60,** 1201 (1938).
[90] F. Schelnk, *Advances in Enzymol.* **9,** 473 (1946).
[91] H. M. Kalckar, *J. Biol. Chem.* **167,** 477 (1947).
[92] J. Wajzer and F. Baron, *Bull. soc. chim. biol.* **31,** 750 (1949).
[93] A. Abrams and H. Klenow, *Federation Proc.* **10,** 153 (1951); *Arch. Biochem. and Biophys.* **34,** 285 (1951).

ing, preparation of ribose-1-phosphate has been effected by the use of this crude system.[93] Klenow and Larsen[94] have recently found that crystalline phosphoglucomutase catalyzes the conversion of ribose-1-phosphate to ribose-5-phosphate and that glucose-1,6-diphosphate acts as a coenzyme. Evidence is also presented to support the view that phosphoribomutase is probably identical with phosphoglucomutase, and, by analogy with the findings of Leloir[95] in connection with the conversion of mannose-1-phosphate to mannose-6-phosphate, it is suggested that the following reaction occurs:

$$\text{ribose-1-P} + \text{glucose-1,6-diP} \rightleftharpoons \text{ribose-1,5-diP} + \text{glucose-6-P}$$

This view is supported by the isolation of ribose-1,5-diphosphate from the reaction products obtained on incubating glucose-1,6-diphosphate and ribose-1-P^{32} with muscle phosphoglucomutase, and the demonstration that it can serve as a coenzyme for both phosphoribomutase and phosphoglucomutase.

b. Phosphodeoxyribomutase

Lampen and his co-workers[96] have shown that the degradation of deoxyribosenucleosides by liver and thymus extracts and by intact cells of *E. coli* is accompanied by the formation of deoxyribose-1-phosphate. This is transformed irreversibly into deoxyribose-5-phosphate by "phosphodeoxyribomutase."

$$\text{hypoxanthine deoxyriboside} + \text{phosphate} \rightarrow \text{deoxyribose-1-P} + \text{hypoxanthine}$$
$$\downarrow$$
$$\text{deoxyribose-5-P}$$

Moderate concentrations of phosphate, sulfate, and arsenate inhibit this mutase.

3. Pentose Isomerases

Two adaptive pentose isomerases have been reported, one in *E. coli*[14] and the other in *Lactobacillus pentosus*,[97] which catalyze the interconversion of D-arabinose and D-ribulose, and D-xylose and D-xylulose, respectively. In both cases, approximately 15% of the keto-sugar is present at equilibrium.

[94] H. Klenow and B. Larsen, *Abstr. 2nd Intern. Congr. Biochem., Paris* p. 235 (1952); *Arch. Biochem. and Biophys.* **37,** 488 (1952).

[95] L. F. Leloir, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 1, p. 67. Johns Hopkins Press, Baltimore, 1951.

[96] L. A. Manson and J. O. Lampen, *J. Biol. Chem.* **191,** 95 (1951); C. E. Hoffmann and J. O. Lampen, *J. Biol. Chem.* **198,** 885 (1952).

[97] J. O. Lampen *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 363. Johns Hopkins Press, Baltimore, 1952.

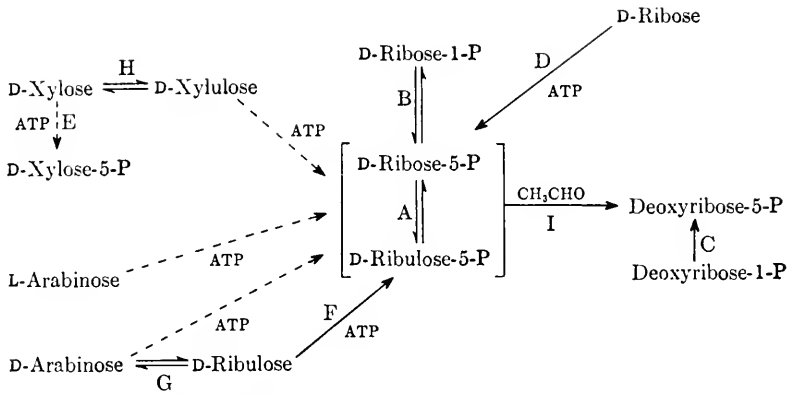

FIG. 7. Interrelationships of pentoses and pentose phosphates. Key to enzymes: A. Phosphoribose isomerase, B. Phosphoribomutase, C. Phosphodeoxyribomutase, D. Ribokinase, E. Xylokinase, F. Ribulokinase, G and H. Pentoseisomerases, I. In presence of pentose phosphate-splitting enzyme and DR aldolase.

These pentose and pentose phosphate interrelationships are shown in Fig. 7.

VII. Metabolism of Pentoses

1. In Microorganisms

a. Oxidation of pentoses

A few bacteria oxidize free pentoses with the formation of the corresponding pentonic acids. This has been reported for certain acetic acid bacteria by Bertrand,[98] who obtained quantitative conversion of xylose to xylonic acid, and by Herman and Neuschul,[99] who obtained pentonic acids from D-arabinose and rhamnose. The formation of pentonic acid from D-arabinose and D-xylose has also been reported for certain *Fusaria*.[100] Higuchi *et al.*[101] found that L-arabinose was oxidized by a strain of *Brucella melitensis* first to arabonic acid and then to the keto acid, which accumulated. With D-xylose, oxidation was more rapid and proceeded beyond the keto acid stage. There is so far no indication that these oxidations involve the formation of phosphorylated intermediates.

Dickens[7,8] investigated the oxidation and fermentation of pentoses and pentose phosphates by yeast extracts and found that only the phosphorylated pentoses were attacked. D-Ribose-5-phosphate was attacked much more readily than either D-arabinose-5-phosphate or D-xylose-5-phosphate, and oxidation was considered to proceed by successive oxidations and decarboxylations through phosphopentonic acid and a tetrose phosphate (see Fig. 1). The observation of Barker and Lipmann[102] that erythritol is phosphorylated and oxidized by propionic acid bacteria offers some sup-

[98] G. Bertrand, *Compt. rend.* **127,** 124 (1898).
[99] S. Herman and P. Neuschul, *Biochem. Z.* **233,** 129 (1931).
[100] A. Hayasida, *Biochem. Z.* **298,** 169 (1938).
[101] K. Higuchi, T. H. Sanders and C. R. Brewer, *Federation Proc.* **10,** 197 (1951).
[102] H. A. Barker and F. Lipmann, *J. Biol. Chem.* **179,** 247 (1949).

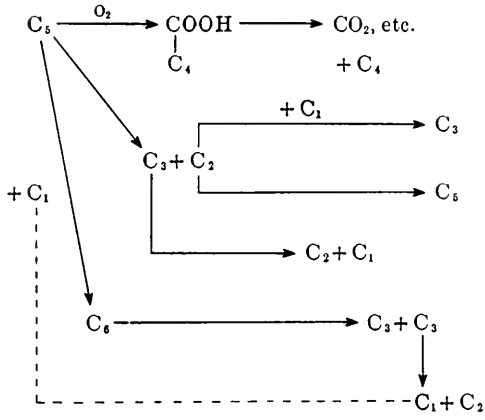

FIG. 8. Methods of pentose metabolism in microorganisms (after Lampen[104]).

port for Dickens' hypothesis. The failure, however, to obtain oxidation of 5-phosphopentonate by partially purified liver enzymes[23] or by leaf extracts[103] probably indicates that oxidative decarboxylation does not proceed beyond the stage of pentose phosphate in plant and animal tissues.

b. Fermentation of Pentose

The general mechanisms which have been proposed for pentose metabolism in microorganisms are shown in Fig. 8.[104] The most generally accepted mechanism is the cleavage into C_2 and C_3 units. A variation of this is the secondary condensation of the C_2 unit either with C_1 to form a C_3 or with other C_2 fragments to give C_6, which could split to C_3 compounds. Addition of C_1 to the C_5 chain to yield C_6, with subsequent cleavage to two C_3 units, has also been suggested.

Considerable evidence exists in support of cleavage into C_2 and C_3 units. Fred *et al.*[105] first reported the formation of equivalent amounts of acetic and lactic acids during fermentation of pentoses by lactic acid bacteria. Dickens[7,8] found that in the presence of DPN, D-ribose-5-phosphate was fermented by yeast extracts producing ethanol, CO_2, inorganic phosphate, and an unknown C_2 compound, indicating preliminary formation of triose phosphate and pyruvate.[106] This is supported by Racker,[74] who showed that extracts of *E. coli* split ribose-5-phosphate into triose phosphate and a C_2 fragment. Many other examples of C_2–C_3 cleavage exist.[21,63-65,107]

The mechanism of fermentation of pentose by lactic acid bacteria has recently been studied with *Lactobacillus pentosus* using D-xylose-1-C^{14} [108] and with *Lactobacil-*

[103] M. Gibbs, *Federation Proc.* **12**, 208 (1953).

[104] J. O. Lampen, *J. Cellular Comp. Physiol.* **41**, 183 (1953).

[105] E. B. Fred, W. H. Peterson and J. A. Anderson, *J. Biol. Chem.* **48**, 385 (1921); E. B. Fred, W. H. Peterson, and A. Davenport, *J. Biol. Chem.* **39**, 347 (1919).

[106] F. Dickens, *Brit. Med. Bull.* **9**, 105 (1953).

[107] R. Kaushal, P. Jowett, and T. K. Walker, *Nature* **167**, 949 (1951); J. Marmur and F. Schlenk, *Federation Proc.* **10**, 221 (1951).

[108] J. O. Lampen, H. Gest, and J. C. Sowden, *J. Bacteriol.* **61**, 97, (1951); H. Gest and J. O. Lampen, *J. Biol. Chem.* **194**, 555 (1952).

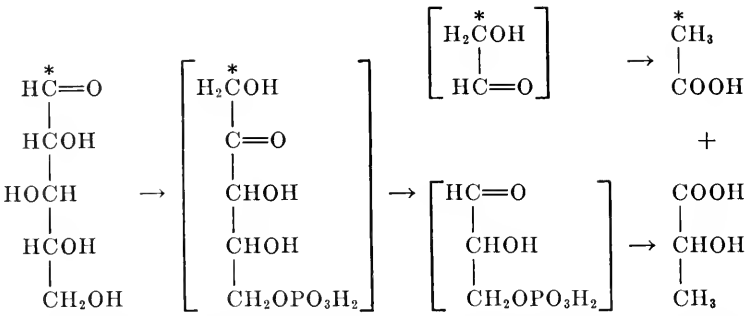

FIG. 9. Suggestive method of degradation of D-xylose-1-C^{14} by *Lactobacillus pentosus* (after Gest and Lampen[108]).

lus pentoaceticus using L-arabinose-1-C^{14}.[109] In both cases, all the C^{14} was found in the methyl group of the acetic acid. This observation that the aldehyde carbon is the source of the methyl carbon of the acetate suggested the intermediary formation of a ketopentose which was believed to be phosphorylated as shown in Fig. 9. When *L. pentosus* was grown on D-xylose or L-arabinose, cells were obtained which fermented D-ribose in addition to these two pentoses.[110] In addition, extracts of these cells degraded ribose-5-phosphate rapidly and catalyzed the phosphorylation of pentoses by ATP.[104] It was suggested that D-xylose and L-arabinose are converted to an ester with the ribose configuration prior to cleavage. This was supported by the formation of ribose-5-phosphate in good yield when D-xylose and ATP were incubated with bacterial extracts, the isolated pentose phosphate fraction containing approximately 80% ribose-5-phosphate with small amounts of ketopentose phosphate and possibly some heptulose phosphate. The mechanism of conversion of xylose to ribose-5-phosphate is obscure. It is possible that a xylose phosphate is the initial product, but xylose-5-phosphate is inert in this system and cannot therefore be an intermediate. It has recently been shown[97] that when D-xylose is incubated with bacterial extracts in the absence of ATP, it is converted into D-xylulose (see Sect. VI.3). This suggests that fermentation of D-xylose possibly involves formation of D-xylulose and subsequent phosphorylation with ATP. Epimerization of the hydroxyl group on C_3 of the hypothetical xylulose phosphate would be necessary to obtain ribose-5-phosphate. These adaptive bacterial enzymes as well as those described by other workers are included in Fig. 7. Adaptive bacterial pentokinases, which catalyze the phosphorylation of pentoses by ATP, have been reported for D-ribose,[111,112] D-arabinose,[111] L-arabinose,[112] D-ribulose,[14] and D-xylose[97,112,113] and suggested, by indirect evidence, for D-xylulose.[97] It is possible that phosphorylation of D-arabinose might consist of initial isomerization to D-ribulose[14] and subsequent phosphorylation of this to ribulose-5-phosphate. A specific ribokinase has also been demonstrated in yeast and the end-product identified as ribose-5-phosphate.[114]

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[109] D. A. Rappoport, H. A. Barker and W. Z. Hassid, *Arch. Biochem. and Biophys.* **31**, 326 (1951).

[110] J. O. Lampen and H. R. Peterjohn, *J. Bacteriol.* **62**, 281 (1951).

[111] S. S. Cohen, D. B. M. Scott, and M. C. Lanning, *Federation Proc.* **10**, 173 (1951).

[112] J. de Ley, *Bull. Assoc. diplômés microbiol. Fac. pharm. Nancy* p. 1 (1952).

[113] R. M. Hochster and R. W. Watson *Abstr. 2nd. Intern. Congr. Biochem., Paris* p. 291 (1952); *Nature* **170**, 357 (1952).

[114] H. Z. Sable, *Biochem. et Biophys. Acta* **8**, 687 (1952).

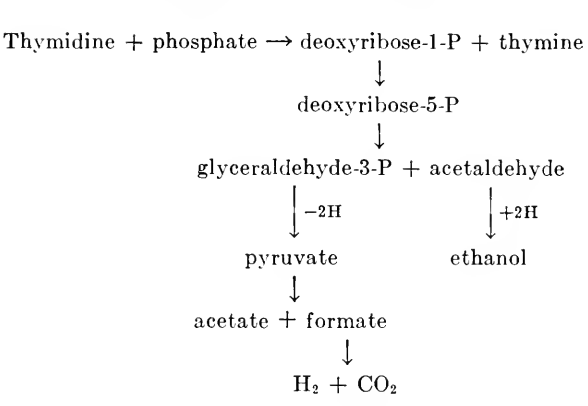

FIG. 10. Suggested pathway of thymidine degradation in *E. coli* (after Lampen[104]).

For a discussion of the evidence indicating the existence of more complex methods of pentose degradation by bacteria, the reader is referred to the review by Lampen.[10] Routes of ethanol formation are discussed by DeMoss.[53]

c. *Fermentation of Deoxyribose*

Very little information is available concerning the fermentation of deoxyribose. Balance studies carried out in Lampen's laboratory[97,115] on the fermentation of thymidine by a strain of *E. coli*, indicated the conversion of 1 mole of deoxyribose to 1 mole each of formate, acetate, and ethanol. Since Racker[76] had already demonstrated the reversible breakdown of deoxyribose-5-phosphate to glyceraldehyde-3-phosphate and acetaldehyde in extracts of *E. coli*, the scheme shown in Fig. 10 was suggested as the probable pathway of thymidine fermentation by this organism. Intact cells of *E. coli* cause no degradation of free deoxyribose.

2. In Mammals

In contrast to many bacteria, it is doubtful whether mammalian tissues can utilize free pentoses. D-ribose is not metabolized by brain cortex slices or erythrocytes,[7] nor D-xylose or D-ribose by rat diaphragm.[116] An increased R.Q. has, however, been reported in guinea pigs after ingestion of D-xylose.[116] It is possible that animal tissues can only metabolize phosphorylated pentoses. So far, however, only one animal pentokinase, catalyzing the phosphorylation of D-xylose by ATP in the presence of rat intestinal mucosa homogenates, has been reported.[117] Extracts of kidney cortex cannot phosphorylate L-xylose, D-ribose, or L-arabinose.[118]

The tolerance of man and most mammals for pentoses is low and when ingested as such they are largely eliminated unchanged in the urine and faeces.[119] In addition to

[115] C. E. Hoffmann and L. A. Manson, *Federation Proc.* **10,** 198, (1951); J. O. Lampen, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 1, p. 160. Johns Hopkins Press, Baltimore, 1951.

[116] N. Northdurft, *Pflügers Arch. ges. Physiol.* **238,** 567 (1937).

[117] M. P. Hele, *Nature* **166,** 786 (1950).

[118] G. E. Youngberg, *Arch. Biochem.* **4,** 137 (1944).

[119] M. Rangier, P. M. de Traverse, and M. Bonvallet, *Bull. soc. chim. biol.* **30,** 583 (1948).

"alimentary pentosuria" resulting from a pentose-rich diet, a rare inborn error of metabolism known as "essential pentosuria" also occurs. In this apparently harmless abnormality, pentose is excreted either as DL-arabinose[120] or more commonly as L-xylulose.[121] Its metabolic significance is unknown although D-glucuronic acid has been suggested as the source of the excreted L-xylulose.[83] Pentosuria, often accompanied by high levels of free or combined pentose in the blood, has also been reported in trauma and shock,[122] in progressive muscular dystrophy,[123] and in experimental hyperthyroidism[124] and is considered to arise from breakdown of tissue ribonucleotides.

Several workers have reported "pentolysis" of added ribose, xylose, and arabinose by blood from humans and rats with malignant tumors,[125] but this has recently been disproved.[126]

VIII. Addendum

The main reactions which participate in the metabolism of pentose phosphates by both animal and plant extracts have been recognised as transaldolisations and transketolisations. Transketolase has been partially purified from rat liver and spinach leaves[127] and catalysis of the following reaction shown to be reversible.

Ribulose-5-P + ribose-5-P $\rightleftharpoons$ sedoheptulose-7-P + glyceraldehyde-3-P.

Pentose phosphate formation is favored at equilibrium. Transketolase is not substrate specific and has already been shown to attack ribulose-5-P sedoheptulose-7-P, L-erythrulose, hydroxypyruvate and fructose-6-P.[27, 127, 128] In all cases cleavage of ketol linkages occurs with the formation of "active glycolaldehyde" which then condenses with an acceptor aldehyde. Ribulose-5-P is formed when glyceraldehyde-3-P is the acceptor.

The formation of hexosemonophosphate from pentose phosphate by rat liver preparations has been studied with C^{14} labeled pentose phosphates.[129] The isotope data indicate that besides the transketolase—transaldolase sequence of reactions involving sedoheptulose-7-P as an intermediate,

[120] C. Neuberg, *Ber.* **33**, 2243 (1900); P. J. Cammidge and H. A. H. Howard, *Brit. Med. J.* **ii**, 777 (1920).

[121] P. A. Levene and F. B. La Forge, *J. Biol. Chem.* **18**, 319 (1914); I. Greenwald, *ibid.*, **88**, 1 (1930); **89**, 501 (1930).

[122] H. N. Green, H. B. Stoner, and M. Bielschowsky *J. Pathol. Bacteriol.* **61**, 101 (1949); W. H. McShan, V. R. Potter, A. Goldman, E. G. Shipley, and R. K. Meyer, *Am. J. Physiol.* **145**, 93 (1945).

[123] A. S. Minot, H. Frank, and D. Dziewiatkowski, *Arch. Biochem.* **20**, 394 (1949).

[124] J. H. Roe and M. O. Coover, *Proc. Soc. Exptl. Biol. Med.* **75**, 818 (1950).

[125] S. N. Steen, *Arch. Biochem.* **26**, 457 (1950).

[126] S. N. Steen, *J. Natl. Cancer Inst.* **12**, 195 (1951); J. H. Roe, J. W. Cassidy, A. C. Tatum, and E. W. Rice, *Cancer Research.* **12**, 238 (1952).

[127] B. L. Horecker, P. Z. Smyrniotis, and H. Klenow, *J. Biol. Chem.* **205**, 661 (1953).

[128] E. Racker, G. de la Haba, and I. G. Leder, *Arch. Biochem. and Biophys.* **48**, 238 (1954).

[129] B. L. Horecker, M. Gibbs, H. Klenow, and P. Z. Smyrniotis, *J. Biol. Chem.* **207**, 393 (1954).

additional reactions must also occur. Similar results were obtained with pea root extracts.[130]

Bloom and his co-workers[131] have attempted to evaluate the relative importance of glycolytic and "oxidative" pathways using glucose-1-C^{14}, glucose-U-C^{14} (uniformly labeled glucose), lactate-1-C^{14}, lactate-2-C^{14}, and lactate-3-C^{14} as substrates. By relating the yields of $C^{14}O_2$ from the labeled lactates to those from the labeled glucoses, the maximal contribution of the glycolytic pathway to the overall conversion of glucose to CO_2 has been calculated. No evidence of a nonglycolytic pathway was found either in the intact rat or in diaphragm sections, whereas in kidney, and more strikingly in liver slices, preferential conversion of C_1 of glucose to CO_2 indicated the occurrence of an alternative pathway. In liver, this alternative pathway accounted for at least 75% of the CO_2 formed from glucose. This somewhat complicated experimental procedure has recently been modified and essentially the same results have been obtained by comparing the yields of $C^{14}O_2$ from glucose-6-C^{14} and glucose-1-C^{14}.[132] The apparent discrepancy between the results for the intact animal and for liver slices may be due to a masking of the liver effect in the whole animal by muscle. The interpretations of these results has been questioned by Katz *et al.*[133] Applying revised equations to the same experimental data they calculated that with rat liver slices, under the special conditions of Bloom *et al.*[131] who incorporated acetate, lactate and gluconate into the medium, only 20% of the CO_2 is derived from glucose via the "oxidative" pathway. Without these additions less than 10% of the CO_2 is formed by this route. Agranoff *et al.*,[134] also using glucose-1-C^{14} and glucose-6-C^{14}, have reported that with rat liver slices there is a shift from the "oxidative" to the glycolytic pathway in regenerating and embryonic liver and in butter yellow carcinomas. Similar results were obtained after fasting and dinitrophenol treatment. Additional work with C^{14} labeled glucose has indicated the participation of the "oxidative" pathway in the metabolism of *Torula utilis*,[135] *Saccharomyces cerevisiae*[136] and various bacteria (see footnote 137). These results with yeast conflict with those of Gilvarg[67] and Chance (see footnote 138). Bernstein[139] has continued his investigations on synthesis of ribose in

[130] M. Gibbs and B. L. Horecker, *J. Biol. Chem.* **208,** 813 (1954).
[131] B. Bloom, M. R. Stetten and DeW. Stetten, Jr., *J. Biol. Chem.* **204,** 681 (1953).
[132] B. Bloom and DeW. Stetten, Jr., *J. Am. Chem. Soc.* **75,** 5446 (1953).
[133] J. Katz, S. Abraham, R. Hill, and I. L. Chaikoff, *J. Am. Chem. Soc.* **76,** 2277 (1954).
[134] B. W. Agranoff, M. Colodzin, and R. O. Brady, *Federation Proc.* **13,** 172 (1954).
[135] J. C. Sowden, S. Frankel, B. H. Moore, and J. E. McClary, *J. Biol. Chem.* **206,** 547 (1954).
[136] H. Beevers and M. Gibbs, *Nature* **173,** 640 (1954).
[137] S. Weinhouse, *Ann. Rev. Biochem.* **23,** 125 (1954).
[138] E. Racker, *Advances in Enzymol.* **15,** 141 (1954).
[139] I. A. Bernstein, *J. Biol. Chem.* **205,** 317, (1953).

the chicken from $CH_3C^{14}OONa$, $C^{14}H_2NH_2COOH$, and $HC^{14}OONa$. The isotope distribution patterns in glycogen and ribose isolated from pooled internal organs were compared, but, since no five consecutive hexose carbons showed the same C^{14} pattern as the ribose isolated from purine nucleotides, it was concluded that neither the direct oxidative pathway nor decarboxylation of uronic acids could account for synthesis of ribose under the given experimental conditions. Although synthesis of ribose by condensation of C_3 and C_2 units is consistent with these data, it is suggested that some as yet unknown mechanism may be involved. Lanning and Cohen[140] consider that the major pathway of ribose synthesis in *E. coli* involves the "oxidative" pathway on account of the low isotope content of the ribose moeity of PNA synthesised when glucose-1-C^{14} was the sole source of carbon.

The formation of ketopentoses from pentitols by DPN-linked polyol dehydrogenases of rat liver and *Acetobacter suboxydans* has been investigated by McCorkindale and Edson.[141] The liver enzyme oxidises xylitol (to D-xylulose) much more readily than ribitol (to D-ribulose) but does not attack D- or L-arabitol, whereas the bacterial enzyme only oxidises D-arabitol (to D-xylulose) and ribitol (to L-ribulose).

Cell-free extracts of *Pseudomonas hydrophila* grown on xylose have been shown to contain a specific xylose isomerase catalyzing the interconversion of D-xylose and D-xylulose.[142] As in the case of the adaptive xylose isomerase of *Lactobacillus pentosus*,[97] approximately 16% of the keto-sugar is present at equilibrium. On account of this active isomerase, Hochster and Watson[142] now consider that the previously reported phosphorylation of D-xylose[113] (see Fig. 7) may actually have been phosphorylation of D-xylulose. Lampen and co-workers,[143,144] have continued their work on the mechanism of formation of ribose-5-P from D-xylose by extracts of *Lactobacillis pentosus*, and the new data confirm their earlier suggestion[97,104] that D-xylose is converted to a ribose (or ribulose) phosphate before degradation of the pentose chain occurs. Since intact cells ferment D-xylulose as rapidly as D-xylose and cell-free extracts phosphorylate D-xylulose very rapidly in the presence of ATP, it has been suggested that the formation of D-xylulose may be the initial step in the fermentation of D-xylose by this organism.[143]

A new enzyme, "phosphoribokinase," catalyzing the phosphorylation of ribose-5-P by ATP to ribose-1,5-diphosphate has been partially purified

[140] M. C. Lanning and S. S. Cohen, *J. Biol. Chem.* **207,** 193 (1954).
[141] J. McCorkindale and N. L. Edson, *Biochem. J.* **57,** 518 (1954).
[142] R. M. Hochster and R. W. Watson, *J. Am. Chem. Soc.* **75,** 3284 (1953).
[143] S. Mitsuhashi and J. O. Lampen, *J. Biol. Chem.* **204,** 1011 (1953).
[144] J. O. Lampen, *J. Biol. Chem.* **204,** 999 (1953).

from pigeon liver.[145] In the presence of adenine and "nucleotide phosphorylase," ribose diphosphate is converted into adenosine monophosphate,[146] thus supporting Greenberg's suggestion[147] that purines can be incorporated directly into nucleotides without prior formation of nucleosides. The coenzyme function of ribose diphosphate in phosphoribomutase and phosphoglucomutase reactions has been reported in some detail.[148]

[145] E. Scarano, *Nature* **172,** 951 (1953).
[146] M. Saffran and E. Scarano, *Nature* **172,** 949 (1953).
[147] G. R. Greenberg, *J. Biol. Chem.* **190,** 611 (1951).
[148] H. Klenow, *Arch. Biochem. and Biophys.* **46,** 186 (1953).

CHAPTER 23

Biosynthesis of Purines and Pyrimidines

PETER REICHARD

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I. Biosynthesis of Purines

1. Synthesis from Small Molecules

Early concepts of the biosynthesis of purines are mainly of historical interest. It has been clear for a long time that higher organisms can synthesize purines *de novo* from other molecules. In Miescher's classical experiments large amounts of nucleic acid in the sperm were synthesized by the male salmon while muscle protein was disappearing. During growth young animals synthesize nucleic acids and nucleotides while exclusively on a milk diet containing only traces of purines. Many similar examples can be found.

a. Biosynthesis of uric acid and hypoxanthine

Uric acid is the chief end-product of all nitrogen catabolism in birds and reptiles. In man and the higher apes relatively small amounts of uric acid are found in the urine, and it has been assumed that in these cases uric acid is more specifically the end-product of purine catabolism (see Chapter 26).

Early studies on the biosynthesis of purines usually involved the classical feeding technique, in which different substances were fed to the fasting animal and the quantitative excretion of uric acid studied. Alanine,[1]

[1] H. B. Lewis, M. S. Dunn, and E. A. Doisy, *J. Biol. Chem.* **36,** 9 (1918).

glycine,[1,2] aspartic[1] and glutamic[1] acids, and pyruvate[3] thus increased the daily output of uric acid and were considered to be possible precursors. Arginine and histidine have also been discussed as possible precursors, mainly because of their similarity to the purines in structure, but also because of a report by Ackroyd and Hopkins[4] that rats excreted a diminished amount of allantoin when put on a diet deficient in these amino acids. Allantoin is the end-product of purine catabolism in the rat (see Chapter 26).

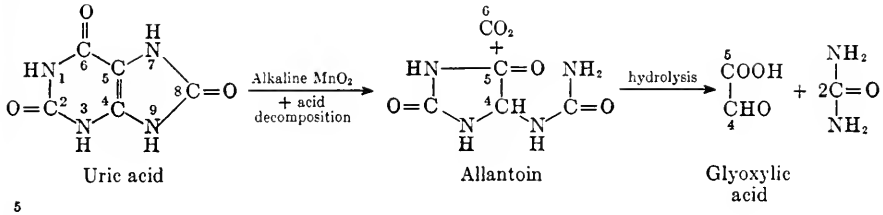
As Lennox[5] has pointed out, all these experiments suffered from the general defect that during the fasting period retention of uric acid by the animal occurred; the uric acid could then be released by the ingestion of the substance under test. The introduction of the isotope technique into this field has made it possible to reinvestigate the problem. Experiments by Bloch and Schoenheimer[6] with arginine-N^{15} proved that the amidine group of this amino acid was not a precursor of allantoin in the rat. Negative results were also obtained by Barnes and Schoenheimer[7] with urea-N^{15}, by Tesar and Rittenberg[8] with histidine-γ-N^{15}, and by Plentl and Schoenheimer[9] with uracil-N^{15} and thymine-N^{15}.

Schuler and Reindel[10] found that pigeon liver slices accumulate a purine-like substance which can be transformed into uric acid by kidney slices. Edson *et al.*[11] identified this substance as hypoxanthine and showed that pigeon liver lacked xanthine oxidase, which was present in kidney slices. These authors furthermore found that the synthesis of hypoxanthine was increased by the addition of lactic and pyruvic acids to the medium. Later Örström *et al.*[12] showed that added oxalacetate and glutamine also had a stimulating effect. Because of the complexity of the system involved, no definite conclusions were drawn, but the possibility of glutamine acting as an "ammonia carrier" was considered.

(1) *Carbon Precursors.* The fundamental work on the carbon precursors of the uric acid excreted by pigeons was carried out by Buchanan and coworkers.[13-16] In their experiments simple metabolically important C^{13}-

[2] A. A. Christman and E. C. Mosier, *J. Biol. Chem.* **83,** 11 (1929).
[3] H. V. Gibson and E. A. Doisy, *J. Biol. Chem.* **55,** 605 (1923).
[4] H. Ackroyd and F. G. Hopkins, *Biochem. J.* **10,** 551 (1916).
[5] W. G. Lennox, *J. Biol. Chem.* **66,** 521 (1925).
[6] K. Bloch and R. Schoenheimer, *J. Biol. Chem.* **138,** 167 (1941).
[7] F. W. Barnes, Jr., and R. Schoenheimer, *J. Biol. Chem.* **151,** 123 (1943).
[8] C. Tesar and D. Rittenberg, *J. Biol. Chem.* **170,** 35 (1947).
[9] A. A. Plentl and R. Schoenheimer, *J. Biol. Chem.* **153,** 203 (1944).
[10] W. Schuler and W. Reindel, *Z. physiol. Chem.* **221,** 209 (1933).
[11] N. L. Edson, H. A. Krebs, and A. Model, *Biochem. J.* **30,** 1380 (1936).
[12] Å. Örström, M. Örström, and H. A. Krebs, *Biochem. J.* **33,** 990 (1939).
[13] J. C. Sonne, J. M. Buchanan, and A. M. Delluva, *J. Biol. Chem.* **166,** 395 (1946).
[14] J. M. Buchanan and J. C. Sonne, *J. Biol. Chem.* **166,** 781 (1946).

Reaction 1



$$\overset{5}{\mathrm{COOH}}\text{—}\overset{4}{\mathrm{CH}}\text{=N—NH—CO—NH}_2 + 3\mathrm{O} \longrightarrow \overset{4}{\mathrm{HCOOH}} + \mathrm{N}_2 + 2\overset{5}{\mathrm{CO}}_2 + \mathrm{NH}_3$$

Reaction 2

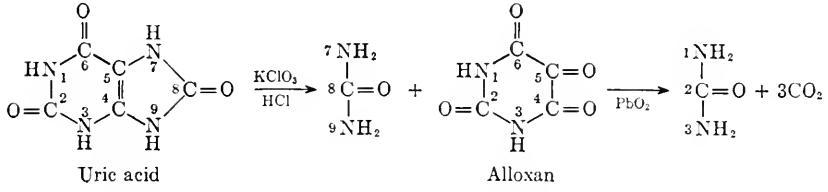


FIG. 1. Degradation of uric acid according to Buchanan *et al.*[16]

labeled substances were administered to pigeons and the uric acid subsequently isolated from the excreta. The uric acid was degraded by previously known reactions and the distribution of the isotope within the molecule determined for each precursor given.

Because of the extreme importance of the degradations in experiments of this type, both here and in the work discussed below, outlines of the degradative procedures are given. The scheme for the degradation of uric acid is represented in Fig. 1.

In the first reaction uric acid was oxidized by the procedure of Edson *et al.*[11] to allantoin and CO_2 (carbon 6), and the allantoin subsequently decomposed to yield urea and glyoxylic acid. Glyoxylic acid was isolated as the semicarbazone and the latter oxidized to give formic acid (carbon 4) and CO_2 (carbon 5).

In the second reaction the method of Liebig and Wöhler[17] was used to oxidize uric acid to alloxan and urea (carbon 8) and the alloxan degraded to CO_2 and urea (carbon 2). A combination of the two reactions permits isolation and isotope analysis of each carbon atom from uric acid.

The results of such degradations after administration of $C^{13}O_2$, $HC^{13}OOH$, $NH_2CH_2C^{13}OOH$, DL-$CH_3CHOHC^{13}OOH$, and DL-$C^{13}H_3C^{13}HOHCOOH$ are summarized in Table I. CO_2 is utilized mainly for the synthesis

[15] J. C. Sonne, J. M. Buchanan, and A. M. Delluva, *J. Biol. Chem.* **173,** 69 (1948).
[16] J. M. Buchanan, J. C. Sonne, and A. M. Delluva, *J. Biol. Chem.* **173,** 81 (1948).
[17] J. Liebig and F. Wöhler, *Ann.* **26,** 259, 262, 312 (1838).

TABLE I

CARBON PRECURSORS IN THE BIOSYNTHESIS OF URIC ACID BY THE PIGEON[15, 16]

Precursor	C^{13} concentration, atom per cent excess						
	Labeled carbon	Uric acid carbon no.					Respiratory CO_2
		2	8	4	5	6	
$C^{13}O_2$	8.13	0.02	0.02	0.07	0.00	0.25	0.28
$HC^{13}OOH$	3.34	2.41	2.41			0.01	0.01
$NH_2CH_2C^{13}OOH$	5.20	0.00	0.00	1.16	0.14	0.11	0.12
DL-$CH_3CHOHC^{13}OOH$	8.80	0.01	0.01	0.37	0.00	0.26	0.25
DL-$C^{13}H_3C^{13}HOHCOOH$	5.40	0.10	0.10	0.07	0.14	0.09	0.11

of carbon 6 of uric acid, formate almost exclusively for carbon 2 and carbon 8, and the carboxyl group of glycine for carbon 4. The incorporation pattern for the two lactic acids can be explained by secondary reactions such as transformation to glycine (via serine), to CO_2, and to formate (via serine and glycine). The initial observation that the carboxyl group of acetate is also a precursor of carbon 2 and carbon 8 has been shown to be erroneous by Elwyn and Sprinson[18] and by Schulman *et al.*[19]

The findings of Buchanan and co-workers have been confirmed and extended in several different laboratories. Karlsson and Barker[20] confirmed the earlier results with $C^{14}O_2$, formate-C^{14} and glycine-1-C^{14}. An investigation with glycine-2-C^{14} showed that although carbon 5 of uric acid contained 52 % of the total activity present, carbon 2, carbon 8, and to a smaller extent carbon 4 of uric acid also contained C^{14}. These findings should be compared with the results of Elwyn and Sprinson,[18] who showed that administration of serine-β-C^{14} causes labeling of the uric acid of pigeons chiefly in positions 2 and 8. According to Sakami[21] α-labeled glycine is converted to α,β-labeled serine. The β-carbon of serine is then transformed into formate or into the same "1-C" derivative that is utilized for the establishment of positions 2 and 8 in uric acid. Other amino acids which can give rise to this formate derivative are L-histidine-2-C^{14} [22] and L-threonine-2-C^{14}.[23] The latter amino acid, however, is converted to α-labeled glycine and therefore contributes most of its label to position 5 in uric acid. A specific role for the whole serine molecule has been postulated by

[18] D. Elwyn and D. B. Sprinson, *J. Biol. Chem.* **184,** 465 (1950).
[19] M. P. Schulman, J. C. Sonne, and J. M. Buchanan, *J. Biol. Chem.* **196,** 499 (1952).
[20] J. L. Karlsson and H. A. Barker, *J. Biol. Chem.* **177,** 597 (1949).
[21] W. Sakami, *J. Biol. Chem.* **176,** 995 (1948).
[22] D. B. Sprinson and D. Rittenberg, *J. Biol. Chem.* **198,** 655 (1952).
[23] A. I. Krasna, P. Peyser, and D. B. Sprinson, *J. Biol. Chem.* **198,** 421 (1952).

Dimroth *et al.*,[24] who found that in *Torula* serine-β-C^{13} contributed more of its isotope to position 6 than to positions 2 and 8 of the purine ring.

Investigations on the carbon precursors of hypoxanthine were initiated by Greenberg[25] with the observation that formate-C^{14} and bicarbonate-C^{14} were incorporated into hypoxanthine by a pigeon liver homogenate. The same author later demonstrated the incorporation of glycine-C^{14} into hypoxanthine by the same system.[26] Greenberg[27] also found that the synthesis of hypoxanthine can proceed in dialyzed extracts of pigeon liver. Furthermore formaldehyde-C^{14}, as well as formate-C^{14}, was incorporated into hypoxanthine in the pigeon liver homogenate, and dilution studies suggested that both substances were converted to a common intermediate before incorporation. Localization of the isotope within the molecule, by degradation of the hypoxanthine, was carried out in none of these cases.

In the meantime Schulman and Buchanan[28] reported independently that particulate-free extracts of pigeon liver can bring about hypoxanthine synthesis. Schulman *et al.*,[19] continuing these experiments, made the very important discovery that the synthesis of hypoxanthine in a nonfortified particulate-free extract of pigeon liver takes place via the reaction of glycine with CO_2 and formate in the molar ratio of approximately 1:1:2. These proportions are good additional evidence that glycine, CO_2, and formate are fundamental carbon units from which hypoxanthine is synthesized.

(2) *Nitrogen Precursors.* The starting point for all further work with simple isotopic molecules was a paper by Barnes and Schoenheimer[7] on the synthesis of purines and pyrimidines from ammonia-N^{15}. Among other things these authors showed that in pigeons an extensive synthesis of uric acid took place from N^{15}-labeled ammonium citrate but they made no attempt to determine the distribution of N^{15} within the uric acid.

Following the work of Sonne *et al.*,[13] Shemin and Rittenberg[29] administered glycine-N^{15} to an adult human male, isolated uric acid from the urine, and, by a partial degradation, determined the amount of N^{15} in positions 1 plus 3, in position 7, and in position 9. It was shown that the greatest amount of N^{15} was located in position 7, and the authors concluded that the N in this position, as well as in positions 4 and 5, was specifically derived from glycine (Table II).

Buchanan *et al.*[16] separated nitrogen atoms 1 plus 3 from nitrogen atoms 7 plus 9 from uric acid obtained after administration of N^{15}-labeled ammonium chloride to pigeons. Equal N^{15} concentrations were observed in

[24] K. Dimroth, E. Jaenicke, and E. W. Becker, *Naturwissenschaften* **39,** 134 (1952).
[25] G. R. Greenberg, *Arch. Biochem.* **19,** 337 (1948).
[26] G. R. Greenberg, *Federation Proc.* **9,** 179 (1950).
[27] G. R. Greenberg, *Federation Proc.* **10,** 192 (1951).
[28] M. P. Schulman and J. M. Buchanan, *Federation Proc.* **10,** 244 (1951).
[29] D. Shemin and D. Rittenberg, *J. Biol. Chem.* **167,** 875 (1947).

TABLE II

INCORPORATION OF GLYCINE-N^{15} INTO THE DIFFERENT NITROGEN ATOMS FROM URIC ACID BY THE HUMAN MALE[29]

Days from start of feeding	N^{15} concentration (atom per cent excess) in uric acid nitrogen no.			
	1 + 3 + 7 + 9	1 + 3	7	9
1	0.078	0.028	0.241	0.015
4	0.459	0.178	1.38	0.100
9	0.308	0.144	0.800	0.144

TABLE III

SYNTHESIS OF URIC ACID BY PIGEONS AFTER ADMINISTRATION OF $N^{15}H_4Cl$ TOGETHER WITH CARBON ISOTOPIC COMPOUNDS[16]

Precursors	N^{15} concentration (atom per cent excess) in uric acid nitrogen atom no.	
	1 + 3	7 + 9
$N^{15}H_4Cl + CH_3C^{13}OOH$	0.856	0.853
$N^{15}H_4Cl + C^{13}H_3C^{13}HOHCOOH$	0.606	0.593
$N^{15}H_4Cl + NH_2CH_2C^{13}OOH$	0.712	0.593

the two groups. However, when glycine-N^{14}-1-C^{13} was administered together with N^{15}-lableled ammonium chloride, positions 1 plus 3 contained more isotope than positions 7 plus 9 (Table III). This was taken as evidence that position 7 in uric acid from pigeons was derived from the nitrogen of glycine.

Elwyn and Sprinson[18] demonstrated that the N^{15} of serine was incorporated into uric acid in pigeons, as might be expected from the well-known transformation of serine into glycine.

A complete degradation, by which the isotope content of each nitrogen of uric acid could be obtained, was described by Lagerkvist.[30] The method is outlined in Fig. 2. Uric acid was methylated to a mixture of 3- and 9-methyluric acids by the method of Fischer.[31] By oxidation with $KClO_3$ the 3-methyluric acid was converted to methylalloxan which, on further oxidation with PbO_2 and hydrolysis with HCl, gave nitrogen atoms 1 and 3 as ammonia and methylamine, respectively. Position 7 of the uric acid was obtained as glycine in a separate procedure by the well-known method of Strecker.[32]

[30] U. Lagerkvist, *Arkiv Kemi* **5**, 569 (1953).
[31] E. Fischer, *Chem. Zentr.* **1897, II**, 157.
[32] A. Strecker, *Z. Chem.* **4**, 215 (1868).

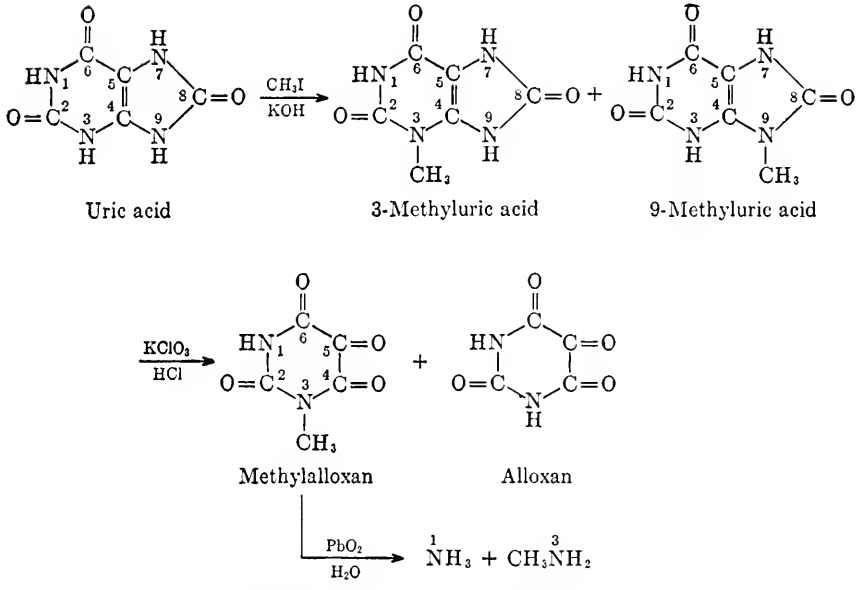


FIG. 2. Degradation of uric acid according to Lagerkvist.[30]

TABLE IV

SYNTHESIS OF URIC ACID FROM $N^{15}H_4Cl$ AND N^{15}-LABELED ASPARTIC ACID IN THE PIGEON[30]

Precursor	N^{15} concentration (atom per cent excess)				
	Labeled nitrogen	Uric acid nitrogen no.			
		1	3	7	9
$N^{15}H_4Cl$	32	0.54	4.51	0.38	4.93
Aspartate-N^{15}	32	1.62	1.59	1.45	2.22

The method was applied to uric acid obtained from pigeons after the administration of N^{15}-labeled ammonium salts and N^{15}-labeled aspartic acid. As shown in Table IV the experiments with ammonia demonstrated a preferential incorporation of the isotope into positions 3 and 9. Aspartate-N^{15} was incorporated into all nitrogen atoms to about the same extent.

The metabolic origin of the nitrogen atoms of hypoxanthine has been investigated by Sonne *et al.*[33-35] using pigeon liver extracts. Use was made

[33] J. C. Sonne and I. Lin, *Federation Proc.* **11**, 290 (1952).
[34] J. C. Sonne and I. Lin, *Federation Proc.* **12**, 271 (1953).
[35] J. C. Sonne, I. Lin, and J. M. Buchanan, *J. Am. Chem. Soc.* **75**, 1516 (1953).

TABLE V

SYNTHESIS OF HYPOXANTHINE IN PIGEON LIVER EXTRACTS FROM DIFFERENT N^{15} PRECURSORS[35]

N^{15} precursor	Moles N^{15} utilized for hypoxanthine synthesis per mole of C^{14}-labeled glycine	N^{15} concentration (atom per cent excess) in hypoxanthine nitrogen no.			
		1 + 3	7 + 9	7	9
NH_4Cl	0.27				
Aspartic acid	1.20	0.091	0.009		
Glutamic acid	1.20	0.185	0.025		
Glutamine (amide N^{15})	1.90	0.186	0.176	0.018	0.334
Glycine	1.00	0.058	0.378	0.750	0.058

of the earlier finding of Schulman *et al.*[19] that in these extracts CO_2, glycine, and formate react in the molecular proportions of 1:1:2 to give one molecule of hypoxanthine. The N^{15}-labeled precursors tested were incubated together with glycine-1-C^{14} in the pigeon liver extracts and the hypoxanthine formed was analyzed for C^{14} and N^{15}. The C^{14} content of hypoxanthine measured the *de novo* synthesis of this purine, and from the ratio of C^{14} to N^{15} in the hypoxanthine the number of N^{15} atoms contributed from each nitrogenous compound to one molecule of hypoxanthine could be calculated. It was found that one atom of N^{15} was contributed from glycine, glutamic acid, or aspartic acid, and two atoms of N^{15} from the amide group of glutamine. By degradation it was shown that the N^{15} from glycine was located in position 7, that from glutamic or aspartic acids in the sum of positions 1 and 3, and those from glutamine in position 9 as well as in the sum of positions 1 and 3 (Table V).

When these results are compared with those obtained by Lagerkvist with ammonia-N^{15} in pigeons it seems probable that the amide group of glutamine is the nitrogenous precursor of positions 3 and 9. Thus aspartic or glutamic acid must donate the nitrogen of position 1. Lagerkvist's results with N^{15}-labeled aspartic acid *in vivo* are probably explained by the many side reactions taking place in such a complex system and by the relative impermeability of the liver to aspartic acid.

Fig. 3 gives a summary of the different carbon and nitrogen precursors for uric acid as far as they are known at the present time.

b. Polynucleotide purines

A paper by Barnes and Schoenheimer[7] contains the first definite indication that the heterocyclic purine molecule can be synthesized from simple molecules. These authors showed that after the administration of N^{15}-

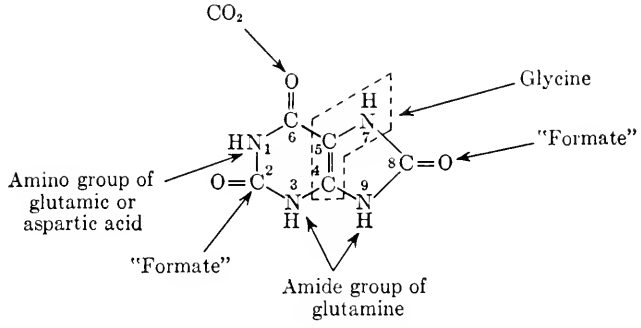


FIG. 3. Precursors of the various atoms in the purine ring of uric acid

labeled ammonium citrate to pigeons or rats the purines from the nucleic acids of the internal organs contained a significant amount of N^{15}. It was further shown by deamination that the amino group of guanine contained more isotope than did that of adenine in relation to the respective ring nitrogens. This metabolic activity of the amino group in position 2 of the purine nucleus, which was later confirmed by Reichard[36] using glycine-N^{15} as precursor in the rat, might evoke speculations as to the possible instability of the carbon in position 2 of the purine ring.

Degradation studies of purines from the nucleic acids of the rat, of pigeons, and of yeast have shown the same biosynthetic pattern for polynucleotide purines as for uric acid. Abrams *et al.*[37] showed that the nitrogen of glycine-N^{15} was preferentially incorporated into position 7 of guanine from the polynucleotides of growing yeast. The biosynthesis of guanine obtained from the mixed nucleic acids of the rat was studied by Heinrich and Wilson.[38] The results showed that positions 2 and 8 were derived from formate-C^{14}, position 6 from $C^{14}O_2$, position 4 from glycine-1-C^{14}, and positions 4 and 5 from glycine-1,2-C^{14} (Table VI). Marsh[39,40] has studied the incorporation of formate-C^{14} into adenine and guanine of nucleic acids from the pigeon. Formate was incorporated into positions 2 and 8 of both purines, and the ratio of the specific activities in the two positions was very nearly 1. These experiments would thus indicate that the purines derive their carbons 2 and 8 from the same common precursor from the same pool. Edmonds *et al.*[41] have found that in yeast formate-C^{14} donates its isotope to positions 2 and 8 of guanine from nucleic acids and that position 5 of this purine was derived from the methyl group of glycine.

[36] P. Reichard, *Acta Chem. Scand.* **3,** 422 (1949).
[37] R. Abrams, E. Hammarsten, and D. Shemin, *J. Biol. Chem.* **173,** 429 (1948).
[38] M. R. Heinrich and D. W. Wilson, *J. Biol. Chem.* **186,** 447 (1950).
[39] W. H. Marsh, *Federation Proc.* **8,** 225 (1949).
[40] W. H. Marsh, *J. Biol. Chem.* **190,** 633 (1951).
[41] M. Edmonds, A. M. Delluva, and D. W. Wilson, *J. Biol. Chem.* **197,** 251 (1952).

TABLE VI

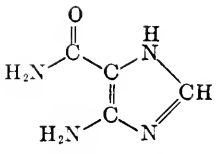
INCORPORATION OF RADIOACTIVE PRECURSORS INTO PURINES FROM POLYNUCLEOTIDES BY THE RAT[38]

C^{14} precursor	C^{14} specific activity, counts/min./mM. carbon						
	Adenine	Guanine	Guanine carbon no.				
			4 + 5	4	6	2	8
CO_2	130	170	35		770	10	0
Formate	4,550	5,760	0			16.400	12.500
Glycine-1-C^{14}	270	300	530	1,220		10	10
Glycine-1,2-C^{14}	65	75	115	135		0	0

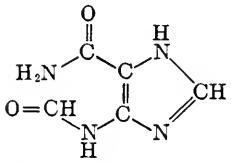
Glycine-2-C^{14} also contributed a considerable part of its label to position 2 (position 8 was not investigated), which is in agreement with the earlier findings of Karlsson and Barker.[20]

The sources of the nucleic acids and the methods of preparation which were used in the above-mentioned studies on the biogenesis of polynucleotide purines make it appear probable that the purines studied were either derived mostly from pentose nucleic acid (PNA) or were obtained from a mixture of PNA and deoxypentose nucleic acid (DNA). No investigations have been reported in which purines from DNA alone have been isolated and degraded after administration of labeled compounds. There is, however, no indication, that the biosynthetic pathway for these purines should differ from that of PNA purines. It has been shown by Bergstrand *et al.*[42] and by Reichard[43] that glycine-N^{15} is utilized for the synthesis of DNA purines in different organs of the rat. LePage and Heidelberger[44] found that glycine-2-C^{14} is incorporated into the DNA purines from rat liver and that this incorporation is of the same order of magnitude as that found in the PNA purines. Elwyn and Sprinson[45] demonstrated that in the rat both glycine-2-C^{14} and serine-3-C^{14} were incorporated into DNA purines to about the same extent as into PNA purines. Totter *et al.*[46] found that formate-C^{14} was utilized by the rat for synthesis of both DNA purines and PNA purines from the viscera. These results have been confirmed by Goldthwait and Bendich.[47]

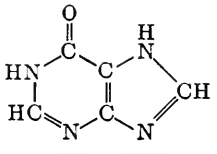
[42] A. Bergstrand, N. A. Eliasson, E. Hammarsten, B. Norberg, P. Reichard, and H. von Ubisch, *Cold Spring Harbor Symposia Quant. Biol.* **13,** 22 (1948).
[43] P. Reichard, *J. Biol. Chem.* **179,** 773 (1949).
[44] G. A. LePage and C. Heidelberger, *J. Biol. Chem.* **188,** 593 (1951).
[45] D. Elwyn and D. B. Sprinson, *J. Am. Chem. Soc.* **72,** 3317 (1950).
[46] J. R. Totter, E. Volkin, and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1521 (1951).
[47] D. A. Goldthwait and A. Bendich, *J. Biol. Chem.* **196,** 841 (1952).



4-Amino-5-imidazole-carboxamide



4-Formylamino-5-imidazole-carboxamide



Hypoxanthine

FIG. 4.

2. The Role of the Carboxamide and the Enzymic Synthesis of Inosinic Acid

By growing *Escherichia coli* in the presence of bacteriostatic amounts of sulfonamides, Stetten and Fox[48] were able to demonstrate the accumulation of a diazotizable amine in the substrate. The amine was isolated but its constitution was not elucidated. Subsequently Shive *et al.*[49] identified this amine as 4-amino-5-imidazolecarboxamide (Fig. 4). These authors proposed that the carboxamide was an intermediate in the biogenesis of purines. In addition to the structural similarity, several other lines of evidence supported this view. The inhibition caused by sulfonamides could, to some extent, be reversed by purines, as was shown by Shive and Roberts.[50] Shive[51] also found that the normal strain of *Lactobacillus arabinosus* could utilize the carboxamide instead of purines, as could a mutant strain of *Ophiostoma multiannulatum*[52] and a hypoxanthine-requiring mutant of *E. coli*.[53] In the latter case the formyl derivative was more active than the unsubstituted carboxamide.

The carboxamide was also accumulated by normal *E. coli* during inhibition with aminopterin[54] and by a purine-requiring mutant of *E. coli*.[55] Recently it has been shown by Stewart and Sevag[56] that the carboxamide is produced by a wild strain of *E. coli B* during normal growth.

Carboxamide-C^{14} was found to be incorporated into the polynucleotide purines and into allantoin of the rat,[57] into hypoxanthine in pigeon liver

[48] M. R. Stetten and C. L. Fox, Jr., *J. Biol. Chem.* **161,** 333 (1945).
[49] W. Shive, W. W. Ackermann, M. Gordon, M. E. Getzendaner, and R. E. Eakin, *J. Am. Chem. Soc.* **69,** 725 (1947).
[50] W. Shive and E. C. Roberts, *J. Biol. Chem.* **162,** 463 (1946).
[51] W. Shive, *Ann. N. Y. Acad. Sci.* **52,** 1212 (1950).
[52] N. Fries, S. Bergström, and M. Rottenberg, *Physiol. Plantarum* **2,** 210 (1949).
[53] R. Ben-Ishai, B. Volcani, and E. D. Bergmann, *Arch. Biochem. and Biophys.* **32,** 229 (1951).
[54] D. W. Woolley and R. B. Pringle, *J. Am. Chem. Soc.* **72,** 634 (1950).
[55] J. S. Gots, *Arch. Biochem.* **29,** 222 (1950).
[56] R. C. Stewart and M. G. Sevag, *Arch. Biochem. and Biophys.* **41,** 9 (1952).
[57] C. S. Miller, S. Gurin, and D. W. Wilson, *Science* **112,** 654 (1950).

homogenates,[58] and into adenine and guanine from nucleic acids of yeast.[59] Williams and Buchanan[60] furthermore isolated a soluble enzyme system from yeast which incorporated the carboxamide-C^{14} into inosinic acid.

The importance of the carboxamide or a derivative of it for the biogenesis of the purines has thus been well established. The problem of ring-closure to form the purine ring (Fig. 4) has received attention by Schulman and Buchanan.[61] It was found that in pigeon liver extracts formate and carboxamide reacted mole for mole to form hypoxanthine. Shive *et al.*[49] had postulated that *p*-aminobenzoic acid or some compound formed from it functions as a coenzyme during ring-closure. Later Rogers and Shive[62] obtained evidence that folic acid might be this coenzyme. The results of Buchanan and Schulman,[63] which will be discussed in more detail below, demonstrated the participation of the citrovorum factor in the enzymic exchange of formate and the carbon in position 2 of the purine moiety of inosinic acid. It is thus probable that the citrovorum factor is closely related to the transformation of the "1-C" derivative to position 2 of the purine ring.

Bergmann *et al.*[64] studied the effect of compounds containing "labile methyl" groups on the accumulation of the carboxamide by sulfadiazine-inhibited *E. coli*. Of all the methyl compounds tested only DL-methionine in the presence of small amounts of *p*-aminobenzoic acid definitely suppressed the accumulation of the carboxamide. Neither formate nor formaldehyde shared this ability. The identification by Cantoni[65] of *S*-adenosylmethionine as an active transmethylating agent might fit these results. Recently Peabody[66] has described the formation in pigeon liver extracts of a nonvolatile formate derivative which was not further described. This compound could be used for the ring-closure to form hypoxanthine.

The problem of whether or not the carboxamide per se is an intermediate in hypoxanthine synthesis has been investigated by Greenberg[67] and by Schulman and Buchanan.[61] Both investigators incubated a labeled precursor (formate-C^{14} and glycine-1-C^{14} respectively) together with an excess of nonlabeled carboxamide in a pigeon liver homogenate. In neither case did the carboxamide contain any activity at the end of the experiment,

[58] M. P. Schulman, J. M. Buchanan, and C. S. Miller, *Federation Proc.* **9,** 225 (1950).
[59] W. J. Williams, *Federation Proc.* **10,** 270 (1951).
[60] W. J. Williams and J. M. Buchanan, *J. Biol. Chem.* **202,** 253 (1953).
[61] M. P. Schulman and J. M. Buchanan, *J. Biol. Chem.* **196,** 513 (1952).
[62] L. L. Rogers and W. Shive, *J. Biol. Chem.* **172,** 751 (1948).
[63] J. M. Buchanan and M. P. Schulman, *J. Biol. Chem.* **202,** 241 (1953).
[64] E. D. Bergmann, B. E. Volcani, and R. Ben-Ishai, *J. Biol. Chem.* **194,** 521 (1952).
[65] G. L. Cantoni, *J. Am. Chem. Soc.* **74,** 2942 (1952).
[66] R. A. Peabody, *Federation Proc.* **12,** 254 (1953).
[67] G. R. Greenberg, *J. Biol. Chem.* **190,** 611 (1951).

nor did it affect the incorporation of C^{14} into hypoxanthine. These experiments indicate that the carboxamide itself is not on the path of hypoxanthine synthesis, but that, instead, a derivative of it is probably involved.

At that time Greenberg[26,27] had already made the important observation that inosinic acid rather than hypoxanthine is the first purine compound formed in pigeon liver systems. This made it possible to explain the results obtained with the carboxamide, and both Greenberg[67] and Schulman and Buchanan[61] suggested that the carboxamide ribotide might be the intermediate in the synthesis of inosinic acid from small molecules and that coupling with ribose phosphate precedes ring-closure. Further support for this theory lies in the tentative demonstration by Ben-Ishai *et al.*[68] of the formation of the carboxamide deoxyriboside from the carboxamide by a cell suspension of *E. coli*, and in the fact that this deoxyriboside had a growth-enhancing effect on a purineless mutant of *E coli*, which was 5 times that of the free carboxamide. Furthermore, Greenberg[69] demonstrated that the carboxamide riboside and not the free carboxamide is the major carboxamide component in *young* cultures of sulfadiazine-inhibited *E. coli*. Recently Greenberg[70] has also shown that in enzyme systems from pigeon liver extracts or from autolysates of brewer's yeast the carboxamide riboside together with phosphoglyceric acid forms the carboxamide ribotide. This ribotide in the presence of formate can then give rise to inosinic acid. On the basis of evidence from trapping experiments with formate-C^{14} and non-labeled carboxamide riboside, Greenberg also suggested that the riboside per se is not an intermediate in the *de novo* synthesis of inosinic acid from small molecules. The combined evidence indicates that ribose and phosphate are attached to an acyclic purine precursor at an early stage prior to carboxamide formation and that it is the carboxamide ribotide which lies on the direct synthetic pathway to inosinic acid (see also Chapter 24).

Studies on the *enzymic synthesis of inosinic acid* have been conducted by Buchanan and co-workers and by Greenberg, and have been of the greatest importance. The earlier-mentioned demonstration by Greenberg[26] that ribose phosphate, or compounds which could give rise to ribose phosphate, stimulated the synthesis of hypoxanthine from formate-C^{14} was the first indication that inosinic acid and not hypoxanthine is the primary purine product formed from small molecules. By following the incorporation of formate-C^{14} into hypoxanthine, inosine, and inosinic acid at different times, the results represented in Fig. 5 were obtained.[67] The figure gives the *total radioactivity* of the compounds. Measurements of the *specific activity* of the same compounds at early stages showed that by far the greater part of

[68] R. Ben-Ishai, E. D. Bergmann, and B. E. Volcani, *Nature* **168,** 1124 (1951).
[69] G. R. Greenberg, *J. Am. Chem. Soc.* **74,** 6307 (1952).
[70] G. R. Greenberg, *Federation Proc.* **12,** 211 (1953).

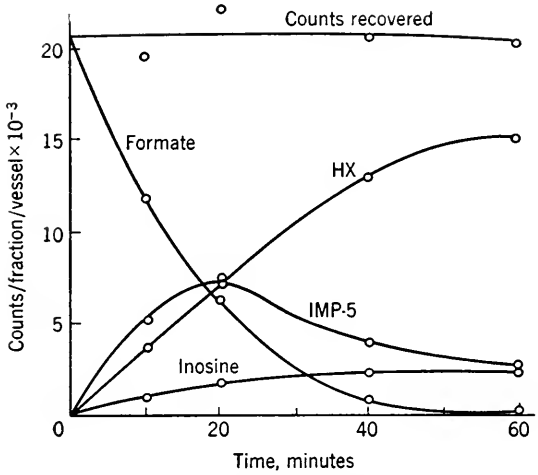


FIG. 5. Incorporation of formate-C^{14} into hypoxanthine (HX), inosine, and inosinic acid (IMP-5) in pigeon liver homogenates at different times (Greenberg[67]).

TABLE VII

RADIOACTIVE FORMATE AS PRECURSOR OF INOSINIC ACID, INOSINE, AND HYPOXANTHINE IN PIGEON LIVER HOMOGENATE[67]

Substance	Specific activity, counts/μM.
Inosinic acid	18,600
Inosine	9,900
Hypoxanthine	4,730

the activity was located in the nucleotide (Table VII) while the nucleoside occupied an intermediate position between inosinic acid and hypoxanthine. Thus inosine could not have been a precursor of inosinic acid but it might well have been an intermediate in the conversion of the nucleotide to hypoxanthine. On the basis of these experiments Greenberg[67] postulated the following reactions for the synthesis of hypoxanthine:

$$CO_2 + 3\ NH_3 + \text{glycine} + 2\ \text{"formate"} + \text{ribose-1-phosphate} \rightarrow \text{inosine-5-phosphate} \xrightarrow{-H_3PO_4} \text{inosine} \xrightarrow{+H_3PO_4} \text{hypoxanthine} + \text{ribose-1-phosphate.}$$

The reversibility of the last two reactions was demonstrated by incubation of a pigeon liver homogenate with labeled hypoxanthine. Isolation of the inosine and inosinic acid formed and analysis of their C^{14} content demonstrated the conversion of hypoxanthine to these substances. Inosine contained almost twice as much C^{14} as did the nucleotide and was therefore considered to be an intermediate in the synthesis of inosinic acid from hypoxanthine.

TABLE VIII

COMPARISON OF RADIOACTIVE INOSINE AND HYPOXANTHINE IN INOSINIC ACID SYNTHESIS BY A PURIFIED ENZYME SYSTEM[71a]

Radioactive substrate	Nonradioactive additions	Radioactivity incorporated into inosinic acid, counts/min.
Inosine	Hypoxanthine, ATP	132
Hypoxanthine	Inosine, ATP	233

The enzyme system involved in the synthesis of inosinic acid from hypoxanthine in pigeon liver has been purified and fractionated by Williams and Buchanan[71,71a] and by Korn and Buchanan.[72] As was pointed out by Buchanan,[73] the study of this reaction should yield very valuable general information on ribotide formation, even though the introduction of ribose and phosphate during the biosynthesis of purines takes place before the purine ring is closed. The reaction which required ribose-5′-phosphate + ATP was catalyzed by at least two enzymes which could be purified by ethanol fractionation. It was shown[72] that none of the enzymes was identical with Kalckar's[74] nucleoside phosphorylase. A comparison between hypoxanthine-C^{14} and inosine-C^{14} with regard to transformation to inosinic acid showed that inosine was not an intermediate in the conversion of hypoxanthine to its nucleotide (Table VIII). The above-mentioned results of Greenberg[67] were explained by postulating the existence of two different independent reactions, one leading from hypoxanthine to inosine and the other from hypoxanthine to inosinic acid.

An interesting observation has been made by Buchanan and Schulman[63] on the participation of the citrovorum factor in the synthesis of inosinic acid. The addition of this factor to pigeon liver extracts enhances the *de novo* synthesis of inosinic acid from glycine. In a system in which nonlabeled inosinic acid had been added at the beginning of the experiment and bicarbonate had been omitted, the citrovorum factor stimulated the incorporation of labeled glycine only slightly but markedly increased the incorporation of formate-C^{14} into position 2 of inosinic acid. A possible scheme for this "enzymic exchange" of formate with position 2 of inosinic acid, as depicted by Buchanan and Schulman, is given in Fig. 6. This problem is also discussed in Chapter 24.

[71] W. J. Williams and J. M. Buchanan, *Federation Proc.* **11**, 311 (1952).
[71a] W. J. Williams and J. M. Buchanan, *J. Biol. Chem.* **203**, 583 (1953).
[72] E. D. Korn and J. M. Buchanan, *Federation Proc.* **12**, 233 (1953).
[73] J. M. Buchanan, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 406. Johns Hopkins Press, Baltimore, 1952.
[74] H. M. Kalckar, *J. Biol. Chem.* **167**, 429 (1947).

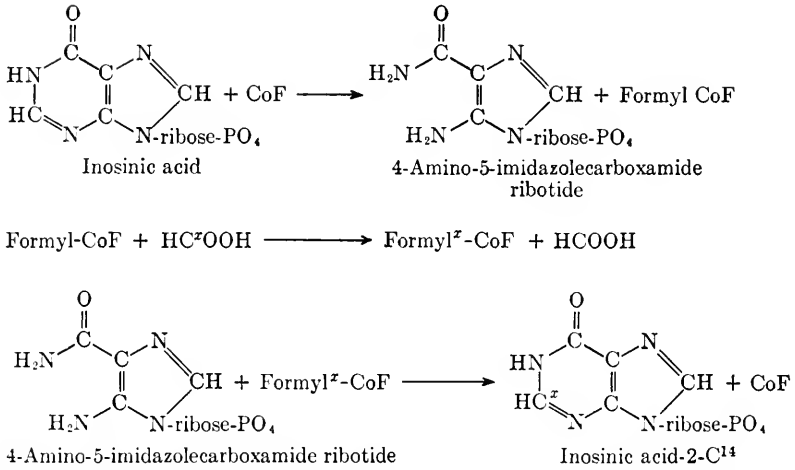
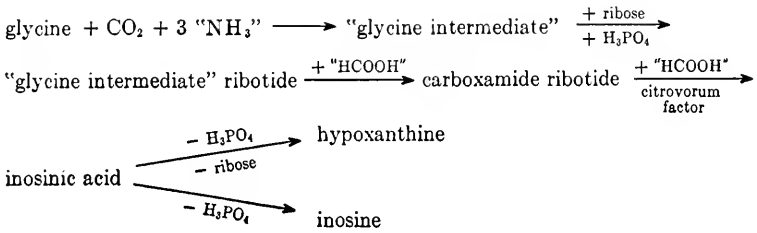


FIG. 6. "Enzymic exchange" of formate with position 2 of inosinic acid.[63] CoF indicates 5,6,7,8-tetrahydrofolic acid; formyl CoF indicates N-5-formyl 5,6,7,8-tetrahydrofolic acid; x designates radioactive carbon. (See also fig. 8 in Chapter 24.)

The following reaction sequence for the synthesis of hypoxanthine summarizes the present experimental evidence:



Many of the reactions are still very obscure, especially those involving the hypothetical "glycine intermediate." This intermediate probably differs from the carboxamide by more than the absence of the carbon in position 8 of the purine ring.[74a] The main purpose of the scheme is to show that ribotide formation takes place before formation of the carboxamide structure.

3. Interconversion of Purines

Although the synthetic reactions leading to the formation of inosinic acid are known to some extent, no link between this substance and the purine bases of nucleic acids has been demonstrated. One cannot conclude a priori that the synthesis of inosinic acid, which in the pigeon eventually

[74a] The present evidence points to glycine-N-amido-5′-phosphoribotide as being the first ribotide compound formed (cf. G. R. Greenberg, *Federation Proc.* **13**, 745 (1954).

leads to the formation of excretory uric acid, must necessarily be involved at all in the synthesis of polynucleotide purines. All comparisons between the small molecule precursors for uric acid and those for polynucleotide purines have, however, shown no differences. Furthermore, the demonstration of the synthesis of inosinic acid in yeast extracts by Williams and Buchanan[60] shows that this nucleotide is synthesized by an organism which does not use the formation of uric acid as an excretory mechanism. The existing evidence thus points to a possible central role of inosinic acid not only in uric acid formation but also in the biosynthesis of polynucleotide purines.

Studies on the enzymic formation of adenosine-5-phosphate, which might be connected with the biosynthesis of the purines of nucleic acids, have indicated that this compound can be formed from adenosine[75] or adenine.[76] The synthesis from adenine-C^{14} was examined in pigeon liver homogenates by Goldwasser,[76] and the formation of isotopic adenosine diphosphate and triphosphate as well as of adenosine-5-phosphate was demonstrated. The formation of acid-soluble radioactive adenine derivatives from adenine-C^{14} by rat liver cells *in vitro* has also been demonstrated by LePage.[77]

A central role of adenine in the biosynthesis of nucleic acid purines was originally postulated by Brown.[78] Brown *et al.*[79] had found that adenine-N^{15} was incorporated into the purines of nucleic acids, while earlier experiments by Plentl and Schoenheimer[9] had demonstrated the nonutilization of guanine-N^{15}. This type of experiment, in which labeled purines, nucleosides, or nucleotides were used as precursors, does not, of course, represent a *de novo* synthesis of the purine ring. Their importance for purine biosynthesis lies in the fact that the results may give indications as to which purine compound is synthesized first from small molecules and may establish a connection between inosinic acid and nucleic acid purines. Such experiments will, therefore, be briefly discussed here although they are dealt with in greater detail in Chapter 25.

The concept that adenine is the first nucleic acid purine formed during biosynthesis in all cases was disputed by Reichard[43] on the basis of the results obtained with glycine-N^{15}. When the incorporation of the isotopic nitrogen into nucleic acid adenine and guanine was studied, in many organs a larger amount of N^{15} was found in the purine ring of guanine than in that of adenine. This was taken as evidence that several biosynthetic pathways leading to polynucleotide purines might exist, one of which did not include the transformation of adenine to guanine.

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[75] A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.* **193,** 481 (1951).

[76] E. Goldwasser, *Nature* **171,** 126 (1953).

[77] G. A. LePage, *Cancer Research* **13,** 178 (1953).

[78] G. B. Brown, *Cold Spring Harbor Symposia Quant. Biol.* **13,** 43 (1948).

[79] G. B. Brown, P. M. Roll, A. A. Plentl, and L. F. Cavalieri, *J. Biol. Chem.* **172,** 469 (1948).

By the use of guanine-C^{14} instead of the labeled-N^{15} compound, it could be demonstrated by Balis *et al.*[80] and by Abrams[81] that the C^{14}-labeled compound was also used by the rat for the synthesis of nucleic acid guanine and adenine, although to a much smaller extent than was adenine. This followed the demonstration that the C 57 black mouse could utilize guanine-N^{15} for synthesis of nucleic acid guanine[82] and that adenine-C^{13} and guanine-C^{14} served equally well as precursors for polynucleotide purines in rabbit bone marrow slices.[83]

Getler *et al.*[84] demonstrated that neither hypoxanthine nor xanthine were precursors of nucleic acid purines in the rat. These compounds had been thought to be hypothetical intermediates in the transformation of adenine to guanine. The results of Abrams and Goldinger,[85] which demonstrate the incorporation of hypoxanthine-8-C^{14} into nucleic acid purines from rabbit bone marrow slices, stress the importance of keeping in mind species differences.

Another possible intermediate, 2,6-diaminopurine, was shown by Bendich *et al.*[86] to be exclusively incorporated into nucleic acid guanine when labeled with either N^{15} or C^{13} and was considered as an intermediate in the transformation of adenine to guanine by the rat.

A wealth of information on different purine utilization patterns can be obtained from microbiological data. For example, many organisms can utilize both guanine and adenine for growth (e.g., yeast,[87] *Lactobacillus casei*,[88] and *E. coli*[89]), others have more specific requirements for adenine[90] which in many cases can be replaced by hypoxanthine,[91] and others specifically require guanine or diaminopurine.[92,93]

The incorporation of free adenine and diaminopurine may not proceed via the corresponding nucleotides, as demonstrated by the results of Roll *et al.*[94] and Kerr *et al.*,[87] who found that the incorporation of the labeled mononucleotides into polynucleotides of the rat and of yeast, respectively, was considerably lower than the utilization of the corresponding free purines. The mononucleotides used in these experiments were not the 5′-nucleotides, however.

The general impression obtained from all the precursor experiments with preformed purines is that a great diversity of biosynthetic patterns may exist. The problem is complicated by the biological heterogeneity of the nucleic acids studied.

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[80] M. E. Balis, D. H. Marrian, and G. B. Brown, *J. Am. Chem. Soc.* **73,** 3319 (1951).

[81] R. Abrams, *Arch. Biochem. and Biophys.* **33,** 436 (1951).

[82] G. B. Brown, A. Bendich, P. M. Roll, and K. Sugiura, *Proc. Soc. Exptl. Biol. Med.* **72,** 501 (1949).

[83] R. Abrams and J. M. Goldinger, *Arch. Biochem.* **30,** 261 (1951).

[84] H. Getler, P. M. Roll, J. F. Tinker, and G. B. Brown, *J. Biol. Chem.* **178,** 259 (1949).

[85] R. Abrams and J. M. Goldinger, *Arch. Biochem. and Biophys.* **35,** 243 (1952).

[86] A. Bendich, S. S. Furst and G. B. Brown, *J. Biol. Chem.* **185,** 423 (1950).

[87] S. E. Kerr, K. Seraidarian, and G. B. Brown, *J. Biol. Chem.* **188,** 207 (1951).

[88] M. E. Balis, D. H. Levin, G. B. Brown, G. B. Elion, H. VanderWerff, and G. H. Hitchings, *J. Biol. Chem.* **196,** 729 (1952).

[89] A. L. Koch, F. W. Putnam, and E. A. Evans, Jr., *J. Biol. Chem.* **197,** 105 (1952).

[90] H. K. Mitchell and M. B. Houlahan, *Federation Proc.* **5,** 370 (1946).

[91] J. G. Pierce and H. S. Loring, *J. Biol. Chem.* **160,** 409 (1945).

[92] G. W. Kidder and V. C. Dewey, *Proc. Natl. Acad. Sci. U.S.* **34,** 566 (1948).

[93] N. Fries, *J. Biol. Chem.* **200,** 325 (1953).

[94] P. M. Roll, G. B. Brown, F. J. Di Carlo, and A. S. Schultz, *J. Biol. Chem.* **180,** 333 (1949).

This has been demonstrated for PNA by many investigators[42,95,96] and for DNA by Bendich *et al.*[97] It is quite possible that this heterogeneity might influence the biosynthesis of purines. The results of Abrams[98] with a purine-requiring yeast mutant might be explained on this basis. He showed that this mutant was still capable of a *de novo* synthesis of PNA purines from glycine-1-C^{14}, even though it required preformed purines in the medium for growth.

Furthermore, Brown *et al.*[99] have shown that the incorporation of adenine into DNA is only a very small fraction of that into PNA in the rat (ratios for DNA-adenine/PNA adenine from 1:29 to 1:73 have been reported), while results by Reichard[36,43] with glycine-N^{15} gave much higher corresponding ratios (1:2 to 1:4). Similarly high ratios with labeled small molecules as precursors have been demonstrated with formate-C^{14},[46] serine-3-C^{14},[45] and glycine-2-C^{14}.[45] In order to rule out the factor of differences in experimental conditions, Furst and Brown[100] administered adenine-C^{14} and glycine-N^{15} simultaneously. The same discrepancy in incorporation ratios was found. Two synthetic pathways for polynucleotide purines seem to exist, one represented by the incorporation of adenine and the other by the *de novo* synthesis of purines from small molecules. This problem is discussed more fully in Chapter 25.

II. Biosynthesis of Pyrimidines

The wealth of data which has accumulated on the biosynthesis of purines greatly exceeds that available for the biosynthesis of pyrimidines. One reason for this is undoubtedly the fact that in pyrimidine biogenesis there is no known substance which can play a role equivalent to that of uric acid and hypoxanthine in the elucidation of purine synthesis. At an early date Cerecedo[101] demonstrated that pyrimidines are catabolized to urea by the dog. These results have been confirmed by Plentl and Schoenheimer,[9] who found that thymine-N^{15} and uracil-N^{15} give rise to urea-N^{15} in the rat. Recently another possible catabolic mechanism in the rat has been found by Fink *et al.*,[102] who demonstrated the excretion of β-aminoisobutyric acid in the rat after the feeding of thymine or DNA. These mechanisms are hardly suited for precursor studies.

On the other hand, the formation of orotic acid in rat liver slices, as demonstrated by Reichard,[103] has proved valuable as a model system for pyrimidine synthesis.[104] The results obtained with this system will be discussed in the section on orotic acid.

[95] R. Jeener, *Nature* **163,** 837 (1949).
[96] C. P. Barnum and R. A. Huseby, *Arch. Biochem.* **29,** 7 (1950).
[97] A. Bendich, P. J. Russell, Jr., and G. B. Brown, *J. Biol. Chem.* **203,** 305 (1953).
[98] R. Abrams, *J. Am. Chem. Soc.* **73,** 1888 (1951).
[99] G. B. Brown, M. L. Petermann, and S. S. Furst, *J. Biol. Chem.* **174,** 1043 (1948).
[100] S. S. Furst and G. B. Brown, *J. Biol. Chem.* **191,** 239 (1951).
[101] L. R. Cerecedo, *J. Biol. Chem.* **88,** 695 (1930).
[102] K. Fink, R. B. Henderson, and R. M. Fink, *J. Biol. Chem.* **197,** 441 (1952).
[103] P. Reichard, *J. Biol. Chem.* **197,** 391 (1952).
[104] P. Reichard and U. Lagerkvist, *Acta Chem. Scand.* **7,** 1207 (1953).

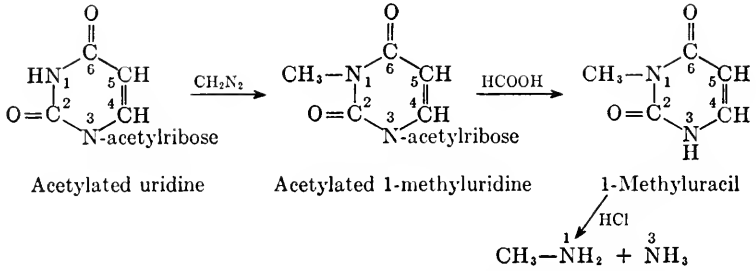

Fig. 7. Degradation of uridine according to Lagerkvist.[105]

1. Small Molecules as Precursors

In their experiments with N^{15}-labeled ammonium citrate, Barnes and Schoenheimer[7] demonstrated the incorporation of the isotope into polynucleotide pyrimidines in pigeons and rats. This, together with the simultaneous demonstration of the N^{15} incorporation into the purines, was the first evidence that polynucleotide compounds were rapidly synthesized from simple precursors.

The results of Bergstrand *et al.*[42] that the isotope from glycine-N^{15} is incorporated into polynucleotide pyrimidines are explained by incorporation via the "nitrogen pool." The intact glycine molecule has been shown not to be involved by Heinrich and Wilson,[38] who could not demonstrate any activity in polynucleotide uracil in the rat after administration of glycine-1,2-C^{14}.

The distribution of N^{15} in the uracil ring after administration of N^{15}-labeled ammonium salts to rats was studied by Lagerkvist.[30] The degradation of the uracil was carried out by a method described earlier by the same author.[105] It consisted in the preparation of the 1-methyl derivative of the acetylated uridine. After hydrolysis to 1-methyluracil, the uracil ring was degraded and position 1 obtained as methylamine, while ammonia represented position 3 (Fig. 7). The results of the degradation after administration of N^{15}-labeled ammonia showed that under the experimental conditions chosen by Lagerkvist the isotope content of position 1 was 3 to 4 times higher than that of position 3.

Investigations on the carbon precursors of polynucleotide pyrimidines showed that position 2 was derived from CO_2 in the rat (Heinrich and Wilson[38]). This finding has been confirmed by Lagerkvist.[106] The report by the latter author that CO_2 is also a specific precursor for carbon 4 of uracil is not valid since the degradation used in these experiments has now been

[105] U. Lagerkvist, *Acta Chem. Scand.* **4**, 543 (1950).
[106] U. Lagerkvist, *Acta Chem. Scand.* **4**, 1151 (1950).

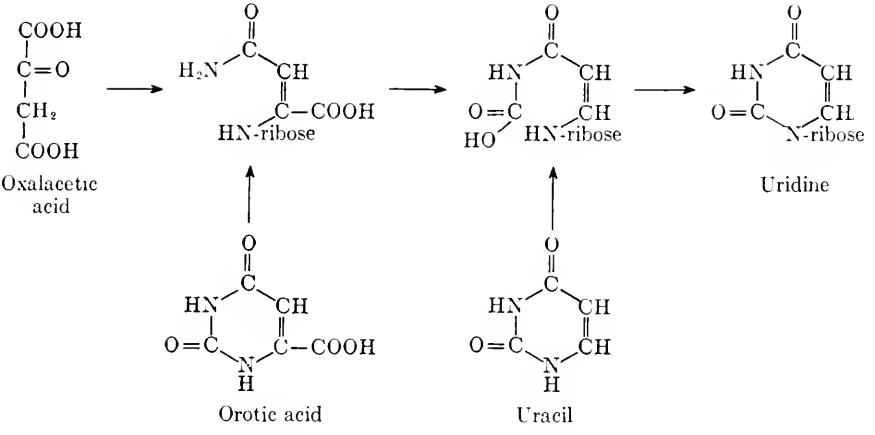

FIG. 8. Biosynthesis of uridine in *Neurospora* according to Mitchell and Houlahan.[109]

shown not to give reliable results for position 4 of uracil.[107] Formate-C^{14} was not found to be a precursor of uracil.[38]

Evidence has been obtained that the 5-methyl group of thymine is derived from a "1-C" derivative. Totter *et al.*[46] found activity in this group using formate-C^{14} as precursor, and Elwyn and Sprinson[45] showed that serine-3-C^{14} and glycine-2-C^{14} also donated their isotope to this methyl group. In contrast to this, LePage and Heidelberger[44] could not find that the isotope was transferred to this group from α-labeled glycine. Elwyn *et al.*[108] suggested that the incorporation of the β-group of serine might not proceed via formate. This suggestion was based on experiments with the β-group labeled with C^{14} and deuterium, in which the results indicated that both atoms were incorporated as a unit. Oxidation of the group to formate would have diluted the deuterium relative to the C^{14} content.

From experiments with mutant strains of *Neurospora*, Mitchell and Houlahan[109] suggested that the carbon chain of the pyrimidine ring could originate from oxalacetic acid. Because of the better utilization of the nucleosides, as compared with the free pyrimidine bases, as growth factors (Loring and Pierce[110]), it was proposed that ribosidation occurred before ring-closure. This theory was very attractive because of the demonstration of a similar situation in the biosynthesis of the purines. However, the experimental evidence in the case of the pyrimidines can be equally well explained on another basis, as discussed below. The reaction sequence proposed by Mitchell and Houlahan[109] is demonstrated by Fig. 8.

[107] U. Lagerkvist, *Acta Chem. Scand.* **7**, 114 (1953).
[108] D. Elwyn, A. Weissbach, and D. B. Sprinson, *J. Am. Chem. Soc.* **73**, 5509 (1951).
[109] H. K. Mitchell and M. B. Houlahan, *Federation Proc.* **6**, 506 (1947).
[110] H. S. Loring and J. G. Pierce, *J. Biol. Chem.* **153**, 61 (1944).

In experiments with *Lactobacillus bulgaricus 09* Wright *et al.*[111] were able to demonstrate that DL-ureidosuccinic acid (labeled with C^{14} in the ureido group) was as effective a precursor for the pyrimidines of nucleic acids as was orotic acid (see below). Orotic acid is required by this bacterium as a growth factor, and cannot be replaced by any pyrimidine derivative.[112,113] Weed and Wilson[114] have shown that this relationship is not limited to *Lactobacillus bulgaricus* by the demonstration that labeled DL-ureidosuccinic acid is incorporated into polynucleotide pyrimidines by the rat.

Bergström *et al.*[115] tested acetylhydantoin-N^{15} and carboxymethylidine-hydantoin-N^{15} in the rat without finding any conversion to polynucleotide pyrimidines. A report by Wright *et al.*[116] that 5-(carboxymethylidine)hydantoin could replace orotic acid as a growth factor for *Lactobacillus bulgaricus 09* was later withdrawn.[111]

Another possible precursor for pyrimidines, aspartate-β-C^{13}-γ-C^{14}, was tested in rat liver slices by Lagerkvist *et al.*[117] The methylene carbon and, to a less extensive degree, the carboxyl group were incorporated into polynucleotide pyrimidines. Aspartate-N^{15} was very poorly utilized. It was tentatively concluded that the carbon chain of aspartic acid was utilized after deamination. Later experiments on the precursors of orotic acid, referred to subsequently, make the interpretation of the results on the N^{15} incorporation improbable. The later results indicated that rapid transamination reactions together with a relatively high permeability barrier for aspartic acid might explain the low incorporation of the N^{15} and that the whole molecule is used as a precursor. It was originally pointed out that the interpretation of the results was rather uncertain since no degradations of the pyrimidines were performed.

A complete degradation of uracil, permitting the isolation of all carbon atoms, was devised by Lagerkvist[107] (Fig. 9). After reduction to hydrouracil, the ring was split by acid hydrolysis, and propionic acid was obtained from the resulting β-alanine via hydracrylic and acrylic acids. The carboxyl carbon of the propionic acid corresponded to position 6 of uracil, the α-carbon to position 5, and the β-carbon to position 4. The propionic acid

[111] L. D. Wright, C. S. Miller, H. R. Skeggs, J. W. Huff, L. L. Weed, and D. W. Wilson, *J. Am. Chem. Soc.* **73,** 1898 (1951).

[112] L. D. Wright, J. W. Huff, H. R. Skeggs, K. A. Valentik, and D. K. Bosshardt, *J. Am. Chem. Soc.* **72,** 2312 (1950).

[113] O. P. Wieland, J. Avener, E. M. Boggiano, N. Bohonos, B. L. Hutchings, and J. H. Williams, *J. Biol. Chem.* **186,** 737 (1950).

[114] L. L. Weed and D. W. Wilson, *J. Biol. Chem.* **207,** 439 (1954).

[115] S. Bergström, E. Hammarsten, and P. Reichard, *Acta Chem. Scand.* **4,** 1497 (1950).

[116] L. D. Wright, K. A. Valentik, D. S. Spicer, J. W. Huff, and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.* **75,** 293 (1950).

[117] U. Lagerkvist, P. Reichard, and G. Ehrensvärd, *Acta Chem. Scand.* **5,** 1212 (1951).

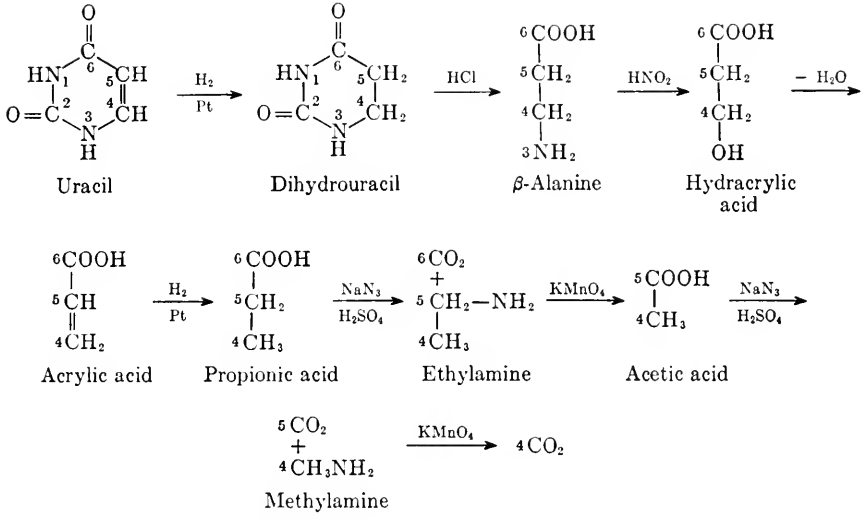

FIG. 9. Degradation of uracil according to Lagerkvist.[107]

was then degraded stepwise according to Phares.[118] In a separate reaction carbon 2 was obtained as urea by degradation of uracil with permanganate. This method was tested by the degradation of uracil-6-C^{14}-5-C^{13}, which gave excellent results.

2. The Function and Biosynthesis of Orotic Acid

Orotic acid (uracil-4-carboxylic acid, Fig. 10) was discovered in milk by Biscaro and Belloni.[119] The simultaneous finding by Loring and Pierce[110] and by Rogers[120] that orotic acid can replace pyrimidines as a growth factor for a mutant of *Neurospora* and for certain streptococci was the first definite indication that this substance might be connected in some way with pyrimidine biosynthesis. Investigations with *Neurospora* mutants were carried on by Mitchell and collaborators.[109,121] From genetic evidence they arrived at the conclusion that orotic acid was not a normal intermediate, but arose in a side reaction during pyrimidine biosynthesis.[121]

An investigation of the possible significance of orotic acid as a pyrimidine intermediate in the rat was carried out by Arvidson *et al.*[122] and by Reich-

[118] E. F. Phares, *Arch. Biochem. and Biophys.* **33,** 173 (1951).

[119] G. Biscaro and E. Belloni, *Ann. soc. chim. Milano* **11,** Nos. 1 and 2 (1905); *Chem. Zentr.* **2,** 64 (1905).

[120] H. J. Rogers, *Nature* **153,** 251 (1944).

[121] H. K. Mitchell, M. B. Houlahan, and J. F. Nyc, *J. Biol. Chem.* **172,** 525 (1948).

[122] H. Arvidson, N. A. Eliasson, E. Hammarsten, P. Reichard, H. von Ubisch, and S. Bergström, *J. Biol. Chem.* **179,** 169 (1949).

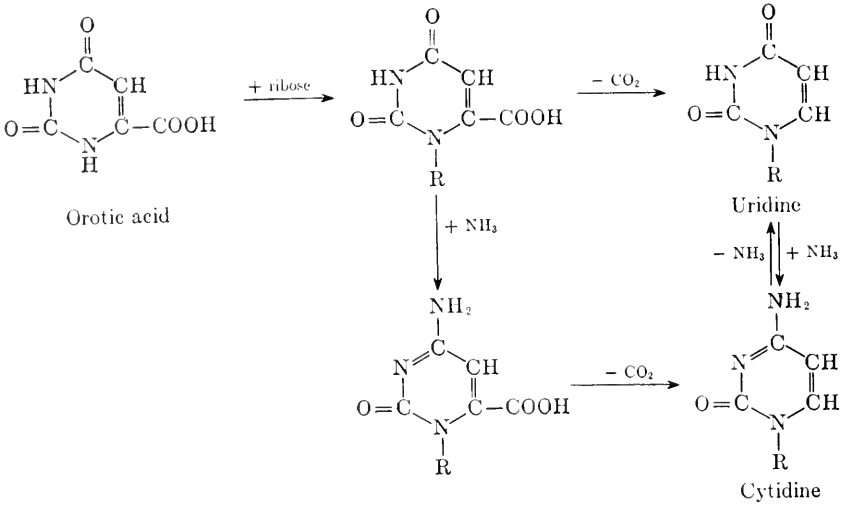

FIG. 10. Possible reactions occurring in the biosynthesis of nucleosides from orotic acid.[122]

ard.[36] It was found that N^{15}-labeled orotic acid was extensively incorporated into the pyrimidines of polynucleotides in different organs while the purines were not labeled (Table IX). Except for a deamination of cytosine no degradations were carried out to prove the utilization of the whole molecule, but the low dilution of the N^{15} seemed to justify the conclusion that this was what had occurred. Later experiments by Weed and Wilson[123] and others with orotic acids labeled in positions 2 and 6 have confirmed the direct utilization. There is a significant incorporation into the pyrimidines of DNA as well as into those of PNA.[36] When DNA/PNA ratios for the incorporations of orotic acid and glycine were compared, however, it was found that the ratio based on glycine incorporation was considerably higher. It is interesting to compare this finding with the corresponding purine ratios for adenine and glycine; in both cases the ratio based on the incorporation of the preformed large molecule was much lower than that based on the *de novo* synthesis from glycine.

The reaction sequence represented in Fig. 10 has been tentatively put forward.[122] Since neither uracil nor cytosine were polynucleotide pyrimidine precursors, it was thought that the attachment of ribose (or ribose phosphate) must precede decarboxylation and that orotic acid riboside (or ribotide) should be an intermediate in the formation of uridine (or uridine phosphate) from orotic acid.

The incorporation of labeled orotic acid into pyrimidines from *E. coli B*,[124]

[123] L. L. Weed and D. W. Wilson, *J. Biol. Chem.* **189**, 435 (1951).
[124] L. L. Weed and S. S. Cohen, *J. Biol. Chem.* **192**, 693 (1951).

TABLE IX

INCORPORATION OF N^{15}-LABELED OROTIC ACID INTO PNA AND DNA FROM DIFFERENT ORGANS OF THE RAT[36,122]

Isolated substances	Liver		Intestines, spleens, and kidneys	
	N^{15}, atom per cent excess	Atom per cent N^{15}, calc. on basis of 100% N^{15} in administered orotic acid	N^{15}, atom per cent excess	Atom per cent N^{15}, calc. on basis of 100% N^{15} in administered orotic acid
From PNA:				
Adenosine	0.011	0.18	0.004	0.07
Guanosine	0.005	0.08	0.006	0.10
Cytidine	0.872	14.4	0.401	6.7
Cytidine ring	—	—	0.592	9.8
Amino group (calc.)			0.029	0.5
Uridine	1.133	18.7	0.324	5.3
From DNA:				
Adenine	0.014[a]		0.005	0.08
Guanine	0.030[a]		0.003	0.05
Cytosine	—		0.091	1.50
Thymine	—		0.062	1.01

[a] The purines from the DNA of liver were contaminated with other nitrogen-containing material as judged by light absorption criteria.

from animal and human tumors,[125] from yeast,[41] and from *Lactobacillus bularicus 09*[111] has now been proved. *In vitro* incorporation of orotic acid in liver slices has been demonstrated by Weed and Wilson[123] and by Reichard and Bergström.[126]

Michelson *et al.*[127] have isolated a nucleoside of orotic acid from the mycelia of *Neurospora*. The point of attachment of ribose on orotic acid was not reported. In contrast to the pyrimidine nucleosides obtained from polynucleotides, this riboside was acid-labile, which might well be explained by the presence of the carboxyl group in position 4. On the other hand, a glycosidic linkage not involving nitrogen 3 of orotic acid might be considered.[128]

[125] L. L. Weed, *Cancer Research* **11**, 470 (1951).

[126] P. Reichard and S. Bergström, *Acta Chem. Scand.* **5**, 190 (1951).

[127] A. M. Michelson, W. Drell, and H. K. Mitchell, *Proc. Natl. Acad. Sci. U.S.* **37**, 396 (1951).

[128] L. D. Wright (personal communication) has found that orotidine cannot replace orotic acid as a growth factor for *Lactobacillus bulgaricus 09*.

TABLE X

INCORPORATION OF $N^{15}H_4Cl$ INTO PNA IN LIVER SLICES WITH AND WITHOUT ADDITION OF NONLABELED OROTIC ACID[103]

Isolated substance	$N^{15}H_4Cl$		$N^{15}H_4Cl$ + nonlabeled orotic acid	
	N^{15}, atom per cent excess	Atom per cent N^{15}, calc. on basis of 100% N^{15} in administered NH_4Cl	N^{15}, atom per cent excess	Atom per cent N^{15}, calc. on basis of 100% N^{15} in administered NH_4Cl
From PNA:				
Adenine	0.323	1.01	0.720	1.11
Guanine	0.185	0.577	0.374	0.575
Uridine	0.284	0.887	0.119	0.183
Cytidine	0.175	0.546	0.208	0.320
From substrate:				
Orotic acid			3.60	5.55

Lieberman and Kornberg[129] have prepared a cell-free extract from an anaerobic bacterium which catalyzed the reactions:

$$\text{orotic acid} \underset{\text{DPN}}{\overset{\text{DPNH}_2}{\rightleftharpoons}} \text{dihydroorotic acid} \rightleftharpoons \text{ureidosuccinic acid} \rightarrow$$

5-acetic acid hydantoin

The demonstrated reversibility of the first two reactions makes it seem possible that this scheme represents the synthetic reactions by which orotic acid is synthesized by the bacteria (*cf.* addendum).

It should be noted that, according to Spicer *et al.*,[130] *Lactobacillus bulgaricus 09* can utilize ureidosuccinic acid but not dihydroorotic acid for growth.[130a]

An attempt to investigate the role of orotic acid as a "natural" intermediate was carried out by Reichard.[103] Rat liver slices were incubated with N^{15}-

[129] I. Lieberman and A. Kornberg, *Federation Proc.* **12,** 239 (1953).

[130] D. S. Spicer, K. V. Liebert, L. D. Wright, and J. W. Huff, *Proc. Soc. Exptl. Med. Biol.* **79,** 587 (1952).

[130a] This "dihydroorotic acid" was synthesized according to Bachstez and Cavallini [*Ber.* **66,** 681 (1933)]. It has recently been found that this substance is probably not identical with dihydroorotic acid. When dihydroorotic acid was synthesized by another chemical method or prepared enzymatically according to Lieberman and Kornberg it could replace orotic acid as a growth factor for *Lactobacillus bulgaricus 09.*[131] *Cf.* also C. S. Miller, J. T. Gordon, and E. L. Engelhardt, *J. Am. Chem. Soc.* **75,** 6086 (1953).

labeled ammonium chloride together with an excess of unlabeled orotic acid, under conditions which permit a synthesis of polynucleotides. It could be shown that the incorporation of isotope from the ammonium chloride was much diminished by the presence of the nonlabeled orotic acid. Furthermore, the orotic acid contained a considerable amount of isotope at the end of the experiment (Table X). Two possible explanations for the experimental results were considered. They are represented by the equations below:

(1) $NH_3 \rightarrow$ orotic acid $\rightarrow$ polynucleotide pyrimidines

(2) $NH_3 \rightarrow$ intermediate $\rightarrow$ polynucleotide pyrimidines
$\downarrow\uparrow$
orotic acid

Further experiments are necessary to distinguish with certainty between these two possibilities, although there is little direct experimental evidence to speak in favor of the more complicated explanation represented by equation (2).

The high N^{15} content of the orotic acid at the end of the experiment indicated a considerable *de novo* formation from ammonia. Much more orotic acid was formed than corresponded to the simultaneous *de novo* synthesis of polynucleotide pyrimidines. It was thought, therefore, that this system should prove valuable for further studies of pyrimidine biosynthesis, and such studies were undertaken by Reichard and Lagerkvist.[104] The general approach was to incubate the labeled precursor and a bank of nonlabeled orotic acid with liver slices, isolate the orotic acid at the end of the incubation, and determine the amount and distribution of isotope within the molecule. In some cases dilution experiments were also carried out, in which the unlabeled precursor was incubated in the system together with N^{15}-labeled ammonium chloride and orotic acid. Again the amount and distribution of N^{15} in the orotic acid was determined at the end of the experiment, and in one case the precursor was also isolated and analyzed for N^{15}.

The degradation of orotic acid is outlined in Fig. 11. After hydrogenation to dihydroorotic acid, the ring was split by acid treatment. Nitrogen 1 was obtained as ammonia nitrogen 3 plus carbons 4 to 7 as aspartic acid. The sum of carbons 6 and 7 was determined as CO_2 by ninhydrin decarboxylation of the aspartic acid, and carbons 4 plus 5 were obtained by difference. Carbon 2 was determined as urea by a separate degradation of the orotic acid with permanganate. The reliability of the method was tested by degradation of orotic acid labeled with N^{15} in position 3.

The precursors investigated were N^{15}-labeled ammonium chloride, bicarbonate-C^{13}, L-aspartate-N^{15}, L-aspartate-1,4-C^{13}, L-aspartate-2,3-C^{14} and L-ureidosuccinic acid (labeled with N^{15} in the nitrogen atom which, after ring-closure, corresponds to position 3 of orotic acid). Dilution experiments

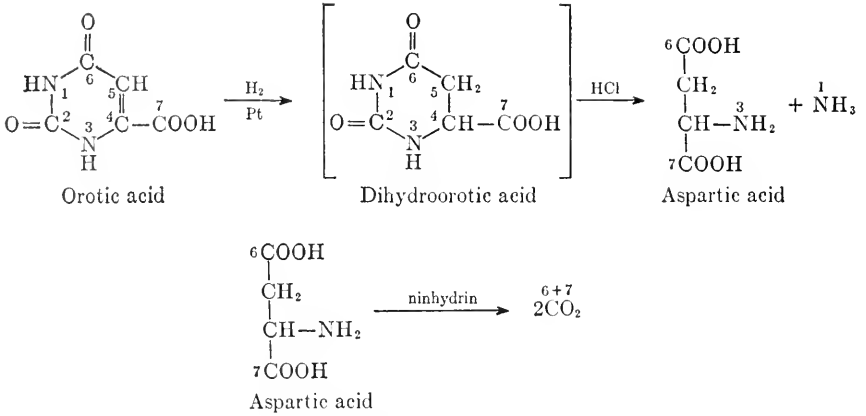

FIG. 11. Degradation of orotic acid according to Reichard and Lagerkvist.[104]

TABLE XI

PRECURSORS IN THE BIOSYNTHESIS OF OROTIC ACID IN LIVER SLICES[a,104]

Isotopic precursor[b]	Hours incubation	Isotope concentration[c] in					
		Orotic acid	N_1	N_3	C_2	$C_6 + C_7$	$C_4 + C_5$[d]
1 mM. L-aspartate-N^{15}	2	0.059[d]	0.012	0.106			
0.5 mM. L-aspartate-1,4-C^{13}	8	0.25			0.04	0.53	0.04
0.5 mM. L-aspartate-2,3-C^{14}	8	129			11	56	261
8 mM. $NaHC^{13}O_3$	6	0.146			0.534		
0.2 mM. L-ureidosuccinate-N^{15}	7	2.70	0.10	5.76			

[a] In each experiment the isotopic precursor was incubated with slices from 3 livers together with 15 mg. orotic acid.

[b] Aspartate-N^{15} = 32 atom % excess; aspartate-C^{13} = 18 atom % excess; $NaHC^{13}O_3$ = 36 atom % excess, ureidosuccinate-N^{15} = 16 atom % excess; aspartate-C^{14} = 8100 counts/min.

[c] Atom per cent excess for N^{15} and C^{13}, counts/min. as $BaCO_3$ at infinitive thickness for C^{14}.

[d] Calculated by difference.

were carried out with L-aspartic acid, DL-ureidosuccinic acid, and L-glutamine. The results are summarized in Tables XI and XII.

In this system ammonia-N^{15} was incorporated equally into positions 1 and 3. The presence of glutamine did not affect this distribution. Aspartic acid, however, under proper conditions, diluted the isotope content of position 3. This, together with the finding that in a shorter experiment the N^{15} from aspartic acid showed up mainly in position 3, makes the amino group of aspartic acid a precursor of nitrogen 3. Unlabeled DL-ureidosuccinic acid considerably diluted the N^{15} incorporation of ammonia into orotic acid. Furthermore, when carrier ureidosuccinic acid was added after incubation in the same experiment, it was found that the ureidosuccinic acid

TABLE XII

BIOSYNTHESIS OF OROTIC ACID IN LIVER SLICES[a, 104]

Isotopic precursor[b]	Nonisotopic addition	Hours incubation	N^{15} concentration (atom per cent excess) in			
			Orotic acid	N_1	N_3	Added nonisotopic compound after incubation
1 mM. $N^{15}H_4Cl$	none	6	2.28			
1 mM. $N^{15}H_4Cl$	1 mM. L-glutamine	6	3.40	3.26	3.31	
1 mM. $N^{15}H_4Cl$	none	1.3	0.67	0.69	0.65	
1 mM. $N^{15}H_4Cl$	3 mM. L-aspartate	1.3	0.28	0.56	0.07	
1 mM. $N^{15}H_4Cl$	none	4	1.71	1.64	1.53	
1 mM. $N^{15}H_4Cl$	0.1 mM. DL-ureidosuccinate	4	0.29			0.082[c]

[a] Experimental conditions as in Table XI.
[b] 32 atom % excess N^{15}.
[c] Isolated after addition of 100 mg. DL-ureidosuccinic acid as carrier.

was labeled with N^{15}. This substance, therefore, seems to be a direct precursor of orotic acid in the liver slice system. This was confirmed by the incorporation of labeled L-ureidosuccinic acid into orotic acid.

Bicarbonate-C^{13} was preferentially incorporated into position 2, as might be expected from the results of Heinrich and Wilson,[38] who showed the corresponding incorporation into uracil.

L-aspartate-1,4-C^{13} donated its isotope almost exclusively to the carbons of positions 6 plus 7, while the label of L-aspartate-2,3-C^{14} showed up mainly in positions 4 plus 5. The relatively low incorporation of the isotopic aspartic acids, compared with ammonia-N^{15} or N^{15}-labeled ureidosuccinic acid, is explained on the basis of permeability barriers for aspartic acid in the liver. In the case of bicarbonate the low incorporation is explained by the continuous exogenous administration of unlabeled CO_2.

A likely reaction sequence for the synthesis of orotic acid is represented by Fig. 12. Nothing is known about the source of the nitrogen for position 1 (*cf.* addendum).

Few data exist on the connection between orotic acid and polynucleotide pyrimidines. Of interest in relation to this is the demonstration by Hurlbert and Potter[131] and by Hurlbert[132,133] of the existence of acid-soluble, radioactive pyrimidine derivatives in rat liver after the administration of

[131] R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.* **195,** 257 (1952).
[132] R. B. Hurlbert, *Federation Proc.* **11,** 234 (1952).
[133] R. B. Hurlbert, *Federation Proc.* **12,** 222 (1953).

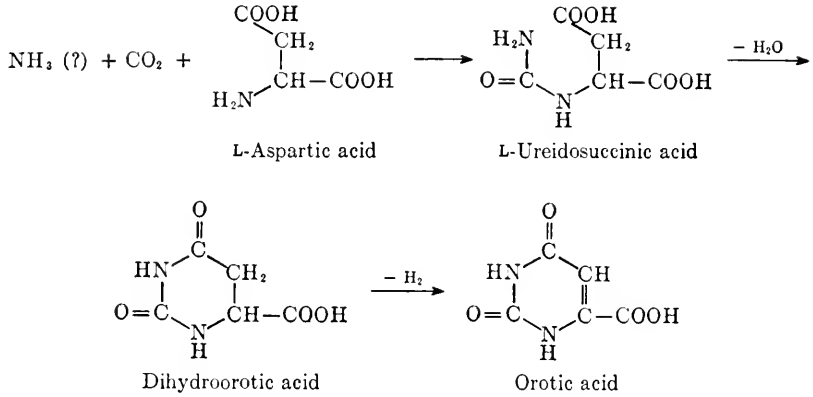

FIG. 12. Biosynthesis of orotic acid.[104]

C^{14}-labeled orotic acid, both *in vivo* and in homogenates. All the fractions so far identified contained uridine plus one or more phosphate groups attached to position 5 of ribose. The higher isotope content of the acid-soluble pyrimidines, compared with that of nuclear PNA pyrimidines, made it possible to consider these uridine phosphates as precursors of PNA. Further work *in vitro* in this direction should prove very interesting (*cf.* addendum).

III. Addendum

The enzymes from *Zymobacterium oroticum* which catalyze the reversible transformation of orotic acid to L-acetic acid hydantoin (5-carboxymethylhydantoin) have been purified and described by Lieberman and Kornberg.[134,135] It was shown that orotic acid is first reduced to L-dihydroorotic acid (*dihydroorotic dehydrogenase*). By ring-opening L-ureidosuccinic acid is formed (*dihydroorotase*), and this acid is then again cyclized to L-acetic acid hydantoin (*5-carboxymethylhydantoinase*). With the exception of the hydantoinase these enzymes are probably also involved in orotic acid formation in the rat. Evidence for dihydroorotic acid being involved in the formation of orotic acid from ureidosuccinic acid (see Fig. 12) in rat liver has been presented by Cooper and Wilson.[136]

The *de novo* synthesis of ureidosuccinic acid in rat liver has been demonstrated by Reichard,[137] who showed that the process is localized in the mitochondria. Ureidosuccinate was synthesized from aspartate, ammonia, and CO_2 in the presence of carbamyglutamate (or acetylglutamate). ATP

[134] I. Lieberman and A. Kornberg, *Biochim. et Biophys. Acta* **12**, 223 (1953).
[135] I. Lieberman and A. Kornberg, *J. Biol. Chem.* **207**, 911 (1954).
[136] C. Cooper and D. W. Wilson, *Federation Proc.* **13**, 194 (1954).
[137] P. Reichard, *Acta Chem. Scand.* **8**, 795, 1102 (1954).

and magnesium ions were also necessary for the reaction to proceed:

$$\text{L-aspartate} + CO_2 + NH_3 \xrightarrow[ATP,\ Mg^{++}]{\text{carbamylglutamate}} \text{L-ureidosuccinate}$$

Several enzymic steps are involved and it seems that the first step—the activation of CO_2 and NH_3—is analogous to the first step in citrulline synthesis.[138] The second step involves condensation of aspartate with the activated intermediate and catalyzation by an enzyme different from the "condensing enzyme" in citrulline synthesis.

Lieberman *et al.*[139] have purified an enzyme (*ureidosuccinase*) from *Z. oroticum* which forms L-aspartate, CO_2, and NH_3 from L-ureidosuccinate. The enzyme requires Mn^{++} or Fe^{++} for full activity and does not seem to be identical with the one described in rat liver.

Evidence for an alternative pathway for pyrimidine biosynthesis in pigeon liver was obtained by Schulman and Badger,[140] who found that the labeled ureido-carbon from L-citrulline was utilized for the synthesis of carbon 2 of the pyrimidine ring both *in vivo* and *in vitro*. This finding might indicate that arginosuccinate is an intermediate in orotic acid formation in pigeon liver.[140a]

The transformation of orotate-C^{14} into uracil derivatives by soluble enzymes was studied independently in Kornberg's laboratory and by Hurlbert and Reichard.[141] Both groups found that uridine-5′-phosphate (UMP) was the first uracil derivative formed from orotic acid, ribose-5′-phosphate, and ATP, and that UMP could be further degraded to uracil and uridine. or phosphorylated to uridine pyrophosphate derivatives.[139,141]

The mechanism of UMP formation from orotic acid has been clarified through the brilliant work of Kornberg and co-workers.[139,142,143] With a purified enzyme from pigeon liver it was shown that 5′-phosphoribosylpyrophosphate (PRPP) is formed from ribose-5′-phosphate and ATP. Orotic acid condenses with PRPP to form orotidine-5′-phosphate, which is then decarboxylated to UMP. These latter two reactions were demonstrated with purified enzymes from yeast. Schematically the whole reaction se-

[138] S. Grisolia *in* "Phosphorus Metabolism," (McElroy and Glass, eds.), Vol. 1, p. 619. Johns Hopkins Press, Baltimore, 1951.

[139] I. Lieberman, A. Kornberg, E. S. Simms, and S. R. Kornberg, *Federation Proc.* **13,** 252 (1954).

[140] M. P. Schulman and S. J. Badger, *Federation Proc.* **13,** 292 (1954).

[140a] An alternative explanation might be that by a reversal of the second step in citrulline synthesis[138] labeled Compound X (the intermediate containing "active" NH_3 and CO_2) is formed from citrulline-C^{14}. Compound X would then condense with aspartate to form labeled ureidosuccinate.

[141] R. B. Hurlbert and P. Reichard, *Acta Chem. Scand.* **8,** 701 (1954).

[142] A. Kornberg, I. Lieberman, and E. S. Simms, *J. Am. Chem. Soc.* **76,** 2027 (1954).

[143] I. Lieberman, A. Kornberg, and E. S. Simms, *J. Am. Chem. Soc.* **76,** 2844 (1954).

quence is represented below:

ribose-5′-phosphate + ATP ⇌ 5′-phosphoribosylpyrophosphate + AMP

PRPP + orotic acid ⇌ orotidine-5′-phosphate + pyrophosphate

orotidine-5′-phosphate → UMP + CO_2

PRPP seems to occupy a central role in nucleotide formation, since this compound together with adenine, guanine, and hypoxanthine in the presence of the proper enzyme fraction forms the corresponding 5′-nucleotides.

Saffran and Scarano[144] had earlier postulated ribose-1,5-diphosphate as an intermediate in the formation of AMP from adenine, ribose-5′-phosphate, and ATP in pigeon liver. It is likely, however, that these authors were studying the same enzymes as Kornberg's group and that also in their case PRPP rather than ribose-1,5-diphosphate was the actual intermediate.

[144] M. Saffran and E. Scarano, *Nature* **172,** 949 (1953).

CHAPTER 24

Biosynthesis of Nucleosides and Nucleotides

F. SCHLENK

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I. Introduction

1. Historical

It is not surprising that an adequate concept of the role of nucleosides or nucleotides in nucleic acid metabolism was not developed by the early in-

vestigators in this field. The primitive state of enzyme chemistry at the turn of the century, and the lack of well-defined nucleic acids and of reliable analytical methods, are responsible for this delay.[1]

Decomposition of nucleic acids into the constituent components was described toward the end of the nineteenth century, and the result was attributed to just one enzyme, nuclease. The complexity of enzyme action in such experiments with crude tissue homogenates or extracts became known much later. The beginning of a systematic mapping of the enzymes as to their range of action and their occurrence in various types of cells dates from the work of Levene[2] and Jones[3] in 1911. Stepwise decomposition into mononucleotides, nucleosides, bases, carbohydrate, and phosphoric acid was found. It seemed logical to assume that the steps observed in nucleic acid degradation constituted the reverse of what happened originally in their biosynthesis. This concept has dominated the thinking of most investigators, and it has remained without challenge until fairly recently.

In general, nucleic acid investigations have followed the trend of other branches of enzymology in the inadvertent emphasis on catabolism rather than anabolism. The first reports of *in vitro* synthesis of nucleosides and nucleotides appeared only a few years ago. Investigations of deamination, dephosphorylation, and similar catabolic reactions still outnumber by far the attempts to bring about the synthesis under controlled conditions. The concept of the reversibility of all these reactions, i.e., the identity of the routes of catabolism and anabolism, may be useful, if we remain ready to change our concepts as future experimental results may call for revisions.

The developments of the type to be discussed in this chapter have gained momentum in recent years; factors responsible for the ever-increasing number of investigations are: the growing interest of many branches of biological science in nucleic acids; the progress in the neighboring field of coenzyme nucleotides; the development of analytical procedures such as tracer techniques, chromatography, and differential spectrophotometry. Further interest has been stimulated by the observation that some vitamins function in enzymatic nucleic acid assembly.

2. Significance of Nucleosides and Nucleotides as Intermediates of Polynucleotide Synthesis

Investigators of the past have assumed that nucleic acid synthesis is the reverse of enzymic disintegration. The sequence suggested was stepwise combination of the bases with ribose or deoxyribose, followed by attachment of phosphoric acid and formation of internucleotide linkages to yield

[1] W. Jones, "Nucleic Acids." Longmans, Green & Co., London, 1920.
[2] P. A. Levene and F. Medigreceanu, *J. Biol. Chem.* **9,** 65, 375, 389 (1911).
[3] W. Jones, *J. Biol. Chem.* **9,** 169 (1911).

polynucleotides.[4] It will be seen from the discussion which follows that little progress has been made in verifying this assumption. Indeed, some noteworthy exceptions to this scheme have been observed, indicating that the ring systems of the bases may be closed after combination with the sugar phosphates. On the other hand, all nucleotides, nucleosides, and the constituent structural units have been obtained by enzymic degradation of nucleic acids, and it would be surprising if one were dealing here with artifacts caused by an idle play of enzymes without biological significance. Provisional acceptance of both possibilities may be advisable. Future research may decide in favor of one of the alternatives or establish multiple pathways. At present, nucleosides and nucleotides will have to be dealt with as intermediates of catabolism and perhaps of anabolism.

II. Biosynthesis of Nucleosides

Early studies of the enzymes resolving the glycosidic bond between ribose (or deoxyribose)and an imidazole or pyrimidine nitrogen were lacking in conclusiveness because of the cumbersome and inaccurate analytical techniques employed. The concept emerged that in degradation the phosphoric acid ester linkage has to be split before nucleosidase action can take place:[4, 5]

$$>\text{N-pentose-PO}_3\text{H}_2 \rightarrow >\text{N-pentose} + \text{H}_3\text{PO}_4 \quad (1)$$

(Nucleotidase action)

$$>\text{N-pentose} \rightarrow >\text{NH} + \text{pentose} \quad (2)$$

(Nucleosidase action)

Gradually, it became apparent that purine and pyrimidine nucleosides are split by different enzymes and that there exists a variety of enzymes in each category.[6]

1. Purine Riboside Phosphorylase

Dixon and Lemberg[7] showed that not all purine nucleosides are split by the enzyme or enzyme complex termed purine nucleosidase by earlier investigators. In particular, the enzyme isolated by them from milk is active on inosine. These authors pointed out that the specificity of nucleosidases is determined mainly by the noncarbohydrate part of the molecule. Levene

[4] P. A. Levene and L. W. Bass, "Nucleic Acids." Chemical Catalog Co., New York 1931.

[5] H. von Euler and E. Brunius, *Ber.* **60**, 1584 (1927).

[6] W. Klein, *in* "Methoden der Fermentforschung" (Bamann and Myrbäck, eds.). Academic Press, New York, 1945.

[7] M. Dixon and R. Lemberg, *Biochem. J.* **28**, 2065 (1934).

and co-workers[8] had already observed that α- or β-methyl-D-ribosides are not split. The restricted action of nucleosidases from various sources, and the importance of the base in determining the susceptibility to the enzymes of this type, have been amply confirmed since Dixon and Lemberg's investigation; some nucleosides with nonbiological bases, however, are an exception.[9]

The studies of the Thannhauser school[10-12] resulted in significant advances. W. Klein[12] extracted and purified purine nucleosidase from spleen, lung, liver, and heart tissue. He found that the enzyme was inactivated by dialysis and reactivated by the addition of phosphate or arsenate. No explanation of the phenomenon was given, and it is not surprising that, without the precedent of any monosaccharide 1-phosphate ester, the nature of the reaction product was not recognized. The method of deproteinizing split most or all of the labile ester, thus allowing determination of the reducing group of the ribose by iodimetry. It remained for H. M. Kalckar[13] to draw the correct conclusions in his continuation of Klein's experiments. With the help of greatly improved and new analytical techniques, which were developed especially for this study, the following reactions were observed to occur with rat liver enzyme:

$$\text{Guanosine} + \text{phosphate} \rightleftharpoons \text{guanine} + \text{ribose-1-phosphate} \qquad (3)$$

$$\text{Inosine} + \text{phosphate} \rightleftharpoons \text{hypoxanthine} + \text{ribose-1-phosphate} \qquad (4)$$

The name *nucleoside phosphorylase* was suggested for this enzyme. The dialysis experiments of Klein[12] could be confirmed with the purified enzyme. With an improved method for the determination of phosphate, which leaves sensitive phosphoric acid esters intact, it was found that inorganic phosphate was esterified to the same extent as the purine base was set free. The structure of ribose-1-phosphate was assigned to this compound. The ester could be isolated and, using it in combination with guanine or hypoxanthine, nucleoside synthesis was achieved (Fig. 1). With equimolar amounts of ribose-1-phosphate and hypoxanthine the equilibrium is 85 to 90% in favor of nucleoside formation. Therefore, if the preparation of ribose-1-phosphate in quantity from inosine or guanosine is required, a high concentration of phosphate is used. Xanthine oxidase, or, if guanosine is used, guanase, is added to deaminate and oxidize the base which is liberated from

[8] P. A. Levene, W. A. Jacobs, and F. Medigreceanu, *J. Biol. Chem.* **11**, 371 (1912).
[9] M. Friedkin, *J. Cellular Comp. Physiol.* **41**, Suppl. 1, 216 (1953); M. Friedkin and D. Roberts, *J. Biol. Chem.* **207**, 245 (1954).
[10] S. J. Thannhauser and M. Angermann, *Z. physiol. Chem.* **186**, 13 (1929).
[11] W. Deutsch and R. Laser, *Z. physiol. Chem.* **186**, 1 (1929).
[12] W. Klein, *Z. physiol. Chem.* **231**, 125 (1935).
[13] H. M. Kalckar, *J. Biol. Chem.* **158**, 723 (1945); **167**, 477 (1947).

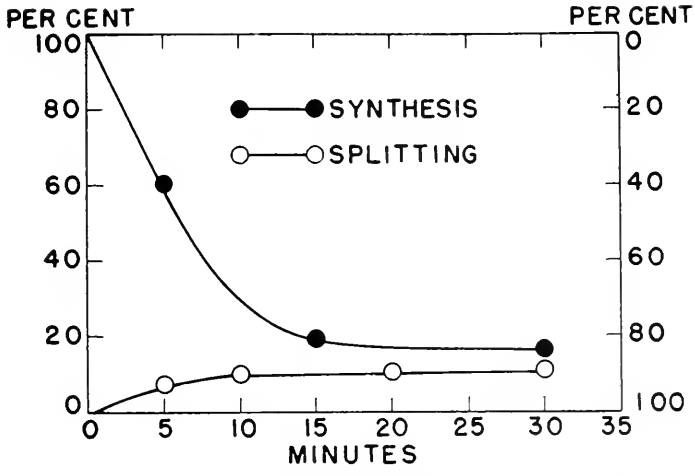


FIG. 1. Splitting and synthesis of inosine.[13] Ordinates: Concentration of substrate mixture in percentage of initial concentration; at the left, ribose-1-phosphate + hypoxanthine: -●—●-; at the right, inosine + phosphate: -○—○-.

the nucleoside.[14] The optical changes which occur during the oxidation of the base are visualized in Fig. 2. In the reactions,

$$\text{Hypoxanthine} \rightarrow \text{Xanthine} \rightarrow \text{Uric Acid} \tag{5}$$

$$\text{Guanine} \rightarrow \text{Xanthine} \rightarrow \text{Uric Acid} \tag{6}$$

the decrease in optical density at 248 mμ is attended by an increase first at 270 mμ (xanthine), and then at 290 mμ (uric acid). This technique has been applied in a micromodification to the investigation of brain tissue.[15]

The limited range of purine nucleoside phosphorylase is illustrated by the lack of action, or insignificantly low effect, on such nucleosides as adenine thiomethylriboside, isoguanosine, and hypoxanthine thiomethylriboside.[16] Korn *et al.*,[17,17a] however, have recently reported that adenine may react with ribose-1-phosphate in the presence of a purified enzyme from beef liver. Friedkin[18,] found that some artificial nucleosides may also be split by nucleoside phosphorylase.[18a]

[14] H. M. Kalckar, *J. Biol. Chem.* **167,** 429 (1947).
[15] E. Robins, D. E. Smith, and R. E. McCaman, *J. Biol. Chem.* **204,** 927 (1953).
[16] M. L. Schaedel, M. J. Waldvogel, and F. Schlenk, *J. Biol. Chem.* **171,** 135 (1947).
[17] E. D. Korn, F. C. Charalampous, and J. M. Buchanan, *J. Am. Chem. Soc.* **75,** 3610 (1953).
[17a] W. J. Williams and J. M. Buchanan, *J. Biol. Chem.* **203,** 583 (1953).
[18] M. Friedkin, *J. Biol Chem.* **209,** 295 (1954).
[18a] I. Lasnitzki, R. E. F. Matthews, and J. D. Smith, *Nature* **173,** 346 (1954).

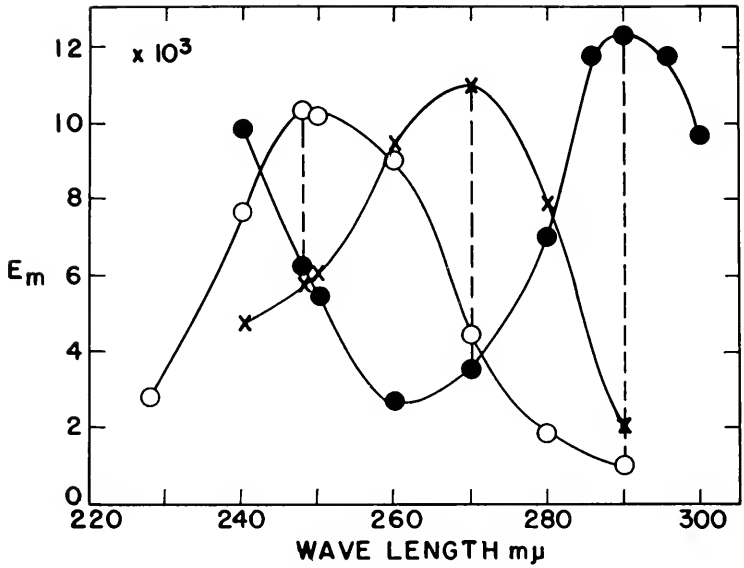


FIG. 2. Absorption spectra of some oxypurines:[14] -○—○-, hypoxanthine; -×—×-, xanthine; -●—●-, uric acid.

2. PURINE RIBOSIDE HYDROLASES

The discovery of the phosphorolysis of purine nucleosides by Kalckar[13] led to the concept that all nucleosides are metabolized according to this mechanism. The first observation to the contrary concerned the hydrolytic cleavage of uridine. More recently, purine riboside hydrolases have been found in bacteria and in yeast, and these do not show the restricted range of action of the phosphorylase.

A comprehensive study of the nucleosidases of yeast was made by Heppel and Hilmoe.[19] The phosphorolytic and hydrolytic enzymes could be separated; the former behaved much like the corresponding enzyme from animal tissues while the latter was active with a variety of purine ribosides such as inosine, guanosine, adenosine, xanthosine, and a number of synthetic nucleosides. Wang and Lampen[20] found in *Lactobacillus pentosus* a hydrolytic enzyme splitting adenosine to adenine and ribose; in addition, inosine, guanosine, xanthosine, and uric acid riboside were hydrolyzed.[21] The crude enzyme was found sensitive to dialysis but, in contrast to the tissue enzyme, phosphate or arsenate did not restore the activity.

[19] L. A. Heppel and R. J. Hilmoe, *J. Biol. Chem.* **198**, 683 (1952).
[20] T. P. Wang and J. O. Lampen, *J. Biol. Chem.* **192**, 339 (1951).
[21] J. O. Lampen and T. P. Wang, *J. Biol. Chem.* **198**, 385 (1952).

3. Purine Deoxyriboside Nucleosidases

In view of the difficulty in the past of securing deoxyribonucleosides, it is not surprising that nucleosidase studies on them are of more recent date than those on ribosides. After the discovery of phosphorolysis of purine ribosides, an analogous nucleosidase reaction with purine deoxyribosides seemed very likely. Proof of this was furnished by Friedkin and Kalckar,[22] who used procedures very similar to those employed in the study of purine ribosides. Purified calf and rat liver[23] and calf thymus[24] nucleosidases were used. These preparations seem to be identical with those acting in the phosphorolysis of purine ribosides; their range of action extends to hypoxanthine and guanine deoxyriboside. In addition to the bases, deoxyribose-1-phosphate was isolated as crystalline cyclohexylamine salt.[25] Experimentation with this ester was complicated by its extreme lability, but Friedkin was able to synthesize hypoxanthine deoxyriboside from it by combination with hypoxanthine in presence of the enzyme. Synthesis is favored if the phosphate concentration is kept low. The identity of the product obtained could be corroborated by microbiological assay.[26] The enzyme from liver shows activity also toward xanthine deoxyriboside, but the rate of reaction is only about $\frac{1}{45}$th of that with deoxyguanosine.[27]

The examination of the nucleosidases from microorganisms has been complicated in some instances by peculiar effects of divalent ions such as phosphate, arsenate, sulfate, or succinate on these enzymes.[28] A stabilizing effect is exerted by these ions on pyrimidine nucleosidase, while the reverse holds for purine nucleosidase from *L. pentosus*, which is rapidly inactivated in their presence. Purine nucleoside phosphorylase, active on deoxyinosine and on deoxyguanosine, has been obtained from *Escherichia coli*.[29] It may also act on deoxyadenosine.[23]

4. Pyrimidine Riboside Phosphorylase

Members of the Thannhauser school did the initial work on pyrimidine nucleosidases. Bone marrow[11] and kidney[12] were used as a source and splitting was accomplished at a slow rate. Various pyrimidine riboside phosphorylases seem to exist, and the work of Klein[12] indicated phosphorolysis rather than hydrolysis.

[22] M. Friedkin and H. M. Kalckar, *J. Biol. Chem.* **184,** 437 (1950).
[23] J. Wajzer, *Arch. sci. physiol.* **1,** 485 (1947).
[24] L. A. Manson and J. O. Lampen, *J. Biol. Chem.* **191,** 95 (1951).
[25] M. Friedkin, *J. Biol. Chem.* **148,** 449 (1950).
[26] E. Hoff-Jørgensen, M. Friedkin, and H. M. Kalckar, *J. Biol. Chem.* **184,** 461 (1950).
[27] M. Friedkin, *J. Am. Chem. Soc.* **74,** 112 (1952).
[28] J. O. Lampen, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 366, Johns Hopkins Press, Baltimore, 1952.
[29] L. A. Manson and J. O. Lampen, *J. Biol. Chem.* **193,** 539 (1951).

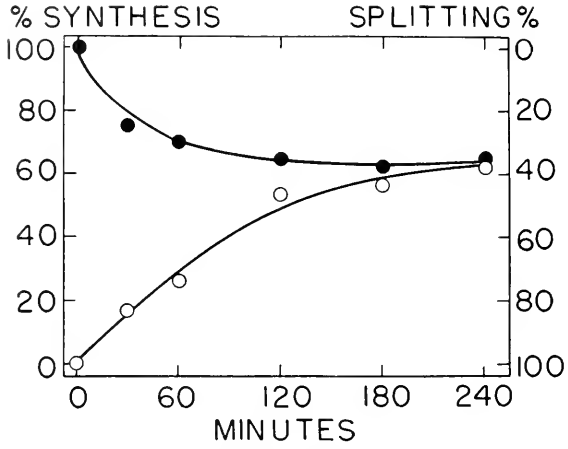


FIG. 3. Equilibrium between enzymic synthesis and splitting of uridine,[31] according to reaction (7). Purified enzyme from *Escherichia coli* was used with a substrate concentration of 4.4 μM. per ml.; synthesis of uridine, -○—○-; splitting of uridine, -●—●-.

A highly purified enzyme was obtained from *E. coli*;[30] it shows specificity for uridine:[31]

$$\text{Uridine} + \text{phosphate} \rightleftharpoons \text{uracil} + \text{ribose-1-phosphate} \qquad (7)$$

Cytidine, purine ribosides, and thymine deoxyriboside are not split, and orotic acid does not react with ribose-1-phosphate in the presence of this enzyme. The study of this reaction was facilitated by a modified orcinol test which permits one to distinguish uracil- and cytosine-bound ribose from other ribosides.[30] The equilibrium attained in reaction (7) is illustrated in Fig. 3.

For the metabolism of cytidine, a connection with uridine by amination or deamination is well established. The recent discovery of orotidine[32] suggests a special enzyme,[32a] bringing about the first step in the utilization of orotic acid which has been observed in many types of cells.

5. Pyrimidine Nucleoside Hydrolases

The discovery of phosphorolytic cleavage of nucleosides[6,13] suggested the ubiquity of this mechanism in nucleoside metabolism. The first exception

[30] L. M. Paege and F. Schlenk, *Arch. Biochem.* **28,** 348 (1950).

[31] L. M. Paege and F. Schlenk, *Arch. Biochem. and Biophys.* **40,** 42 (1952).

[32] A. M. Michelson, W. Drell, and H. K. Mitchell, *Proc. Natl. Acad. Sci. U. S.* **37,** 396 (1951).

[32a] I. Lieberman, A. Kornberg, E. S. Simms, and S. R. Kornberg, *Federation Proc.* **13,** 252 (1954).

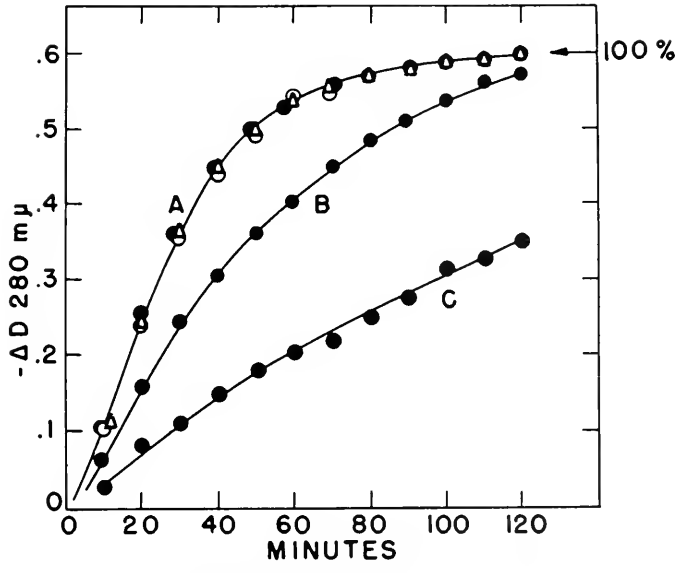


FIG. 4. Assay of the enzymic hydrolysis of uridine by spectrophotometry;[33] 76 μg. of uridine per ml.; -●—●-, phosphate; -△—△-, glycine; -○—○-, arsenate buffer (0.1 *M*, pH 7.0). Curves A, B, and C: 125, 63, and 32 μg. of enzyme protein per ml.; $t = 26°$.

to such a generalization was reported by Carter.[33] He purified an enzyme from plasmolyzed yeast by ammonium sulfate fractionation and found that it degrades uridine to uracil and ribose (Fig. 4). Inorganic phosphate or arsenate is not needed in this process, which goes to completion. The reaction was measured by ultraviolet spectrophotometry as shown in Fig. 5. A well-defined optimum for activity was found at pH 7.0 in phosphate, glycine, and veronal buffers. The reaction follows first-order kinetics up to 83% hydrolysis of the substrate. An excess of uracil and, to a lesser extent, ribose was found inhibitory to the splitting of uridine. Adenosine, inosine, guanosine, cytidine, and thymidine were not degraded by this enzyme. Uridylic acid was not split, nor did it inhibit the degradation of uridine. Reversibility of this process has not been reported, but Horecker[36] believes that this may be due to the high concentration of water in the medium compared with the phosphate concentration in phosphorolysis; the equilibrium constants for these two reactions may be nearly the same, but in presence of such an excess of water the reversal cannot be observed.

[33] C. E. Carter, *J. Am. Chem. Soc.* **73,** 1508 (1951).
[34] R. D. Hotchkiss, *J. Biol. Chem.* **175,** 315 (1948).
[35] H. M. Ploeser and H. S. Loring, *J. Biol. Chem.* **178,** 431 (1949).
[36] B. L. Horecker, *J. Cellular Comp. Physiol.* **41,** Suppl. 1, 279 (1953).

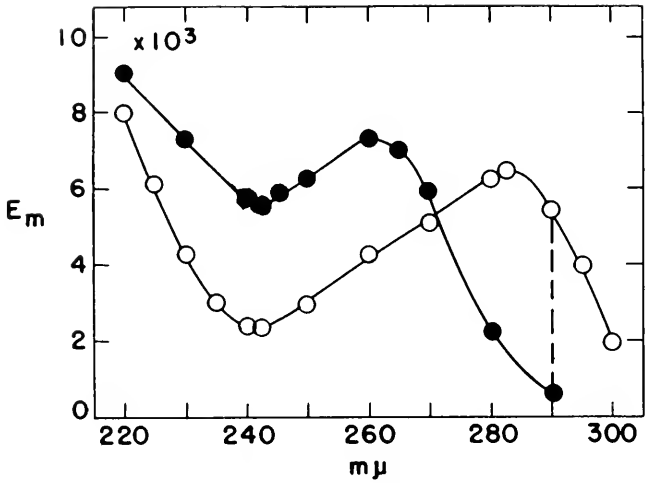


FIG. 5. Absorption spectrum of uracil, -○—○-, and uridine, -●—●-, at pH 11.5. The enzymic splitting of uridine can be measured by adjustment of aliquots of the incubation mixture with alkali and observation of the increase in density at 290 mμ. An alternative is the continuous observation of the density at 280 mμ in neutral medium.[33-35]

Lampen and Wang[21] found a nucleoside hydrolase in extracts from *L. pentosus* which hydrolyzed cytidine and uridine. Divalent ions such as HPO_4^{--}, $HAsO_4^{--}$, SO_4^{--}, or succinate stimulated the reaction. Lampen[28] believes that these ions increase the stability of the pyrimidine nucleosidase which otherwise is completely inactive after one minute at 37° C. Free sugar and base are the products of the reaction.

6. Pyrimidine Deoxyriboside Phosphorylase

The occurrence of pyrimidine deoxyriboside nucleosidase in kidney was observed by W. Klein.[12] The preparations of Wajzer[23] and of Friedkin and Kalckar[22] act on various deoxyribose nucleosides, and the preparation from horse liver[9] catalyzes the following reactions:

$$\text{Thymine} + \text{deoxyribose-1-phosphate} \rightleftharpoons \text{thymine deoxyriboside} + \text{phosphate} \quad (8)$$

$$\text{Uracil} + \text{deoxyribose-1-phosphate} \rightleftharpoons \text{uracil deoxyriboside} + \text{phosphate} \quad (9)$$

According to Friedkin[9] the protozoon *Tetrahymena geleii* is another suitable source of this enzyme. Pyrimidine deoxyriboside phosphorylase from *E. coli* has been studied by Manson and Lampen.[29] The specificity of this enzyme was the same as that of the tissue enzyme. Cytosine deoxyriboside was not split.

There are as yet no studies on record concerning 5-methylcytosine deoxy-

riboside; future research will have to deal also with orotic acid deoxyriboside and 5-methylorotic acid deoxyriboside, if such nucleosides should be discovered. It is of interest to note that 5-methylorotic acid (thymine-4-carboxylic acid) was not found superior to thymine in tissue nucleic acid incorporation.[37,37a]

7. Nucleoside-*N*-transglycosidases

The discovery of nucleoside-*N*-transglycosidase stems from bacterial growth experiments. It was found that several of the deoxyribose nucleosides can substitute for the vitamin B_{12} requirement of certain bacterial species.[38,39] Since it is apparently immaterial which of the deoxyribose nucleosides is provided in the growth medium, one must assume that a mechanism for rapid interconversion exists to meet the needs of the cells for deoxyribonucleic acid synthesis. This question was studied by McNutt,[40] who found nucleoside-*N*-transglycosidase action to be the underlying principle. From earlier observations it seemed likely that the interconversion of one deoxyriboside into another involves two steps:

$$N_1\text{-deoxyriboside} + \text{phosphate} \rightleftharpoons N_1 + \text{deoxyribose-1-phosphate} \quad (10)$$

$$N_2 + \text{deoxyribose-1-phosphate} \rightleftharpoons N_2\text{-deoxyriboside} + \text{phosphate} \quad (11)$$

If this mechanism were correct, deoxyribose-1-phosphate together with the requisite bases should have substituted for the deoxyribonucleosides in promoting bacterial growth; this, however, was not the case nor was phosphate needed. Likewise, it was possible to exclude a hydrolytic mechanism:

$$N_1\text{-deoxyriboside} \rightleftharpoons N_1 + \text{deoxyribose} \quad (12)$$

$$N_2 + \text{deoxyribose} \rightleftharpoons N_2\text{-deoxyriboside} \quad (13)$$

Purine and pyrimidine bases with added deoxyribose failed to substitute for deoxyribose nucleosides in growth tests. It became clear, therefore, that the organisms examined (*L. helveticus*, *L. delbruckii*, and *Thermobacterium acidophilum*) contain one or several enzymes to bring about the interconversions. The name *nucleoside-N-transglycosidase* has been proposed.[40]

The analytical procedures in the study of this new enzyme included paper chromatography, ultraviolet spectrophotometry, and a differentiation between purine and pyrimidine deoxyribosides based on the relative resistance of the latter toward acid. Thus, starting with a purine deoxyriboside and a

[37] G. B. Brown, P. M. Roll, and H. Weinfeld, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 385. Johns Hopkins Press, Baltimore, 1952.

[37a] W. L. Holmes and W. H. Prusoff, *J. Biol. Chem.* **206**, 817 (1954).

[38] V. Kocher and D. Schindler, *Intern. Z. Vitaminforsch.* **20**, 441 (1949).

[39] E. Kitay, W. S. McNutt, and E. E. Snell, *J. Biol. Chem.* **177**, 993 (1949).

[40] W. S. McNutt, *Biochem. J.* **50**, 384 (1953).

pyrimidine base, the formation of the pyrimidine deoxyriboside could be followed by acid hydrolysis of the mixture. Any purine deoxyriboside remaining at the end of the incubation period is readily hydrolyzed, while the pyrimidine deoxyriboside is relatively resistant and can be discovered by paper chromatography or by its bacterial growth promoting ability.[41]

With dialyzed enzyme from *L. helveticus*, and with hypoxanthine deoxyriboside as the donor of the carbohydrate moiety, thymine, uracil, cytosine, and 5-methylcytosine accepted the deoxyribosyl group yielding pyrimidine deoxyribose nucleosides. In every instance, more than half of the purine deoxyriboside was converted to pyrimidine deoxyriboside if an excess of the pyrimidine base was provided. Transglycosidation of deoxyribose from one purine to another purine or from one pyrimidine to another was also observed. Likewise, transfer of the deoxyribose from pyrimidine nucleosides to purine bases was accomplished. The following compounds were found reactive with thymine deoxyriboside: adenine, guanine, hypoxanthine, xanthine, and 4-amino-5-imidazolecarboxamide. Uric acid and 2,6-diaminopurine did not enter into the exchange reaction. All these observations were greatly facilitated by the fact that the rather crude bacterial enzyme preparations were relatively free from deoxyribo- (but not ribo-) nucleosidases and from deaminases. The discovery of uracil deoxyriboside by McNutt[40] among the reaction products of this enzyme system deserves special consideration. This compound may be a precursor of both cytosine and thymine deoxyribosides.

In cases where transfer of deoxyribose from one purine to another or from one pyrimidine to another is observed, a transglycosidation might be simulated by deamination, transamination, oxidation, or reduction of the base with the sugar remaining linked to it. Thus, the formation of adenine deoxyriboside from adenine and hypoxanthine deoxyriboside could be the result of a transamination[42] shifting the 6-amino group of the free adenine to hypoxanthine deoxyriboside (equation a) or the result of transglycosidation (equation b). These alternatives can be tested by the use of labeled material:

(a) Adenine-8-C^{14} + hypoxanthine deoxyriboside $\rightleftharpoons$ hypoxanthine-8-C^{14} + adenine desoxyriboside.

(b) Adenine-8-C^{14} + hypoxanthine deoxyriboside $\rightleftharpoons$ adenine-8-C^{14} deoxyriboside + hypoxanthine.

Similar formulations are possible for other purines and pyrimidines and their deoxyribosides. Kalckar and co-workers[43] tested the alternatives formulated

[41] E. Hoff-Jørgensen, *Biochem. J.* **50,** 400 (1950).
[42] M. Stephenson and A. R. Trim, *Biochem. J.* **32,** 1740 (1938).
[43] H. M. Kalckar, W. S. McNutt, and E. Hoff-Jørgensen, *Biochem. J.* **50,** 397 (1952).

above and verified the transglycosidation mechanism. Exchange of adenine bound in adenine deoxyriboside with free adenine-8-C^{14} was also observed.

There is no indication as yet that enzymes of this type function in nucleic acid synthesis of higher animals. A study of the analogous transfer of the ribosyl group in bacteria and other organisms is needed;[43a] a complicating factor is the apparent lack of suitable test organisms requiring ribonucleosides for growth, and the presence of powerful ribonucleosidases in bacterial preparations of the type described above. These difficulties could be overcome by using induced mutants requiring ribose nucleosides for growth.

Some observations by Kritskiĭ[44] may be interpreted as transribosidation; this author, however, assumes a special mechanism in which phosphoric acid ion, bound to the enzyme protein, comes into play.

8. Interconversion of Nucleosides and Nucleotides by Reactions other than Transglycosidation

Experimental data and observations suggest that the nucleosides and nucleotides in most cells are not assembled individually; rather, the production may be restricted to one compound each of the pyrimidine and purine type. Others are produced from these as needed by oxidation and amination. The changes occurring are confined to the nitrogenous bases; depending on the type of cells or tissue, they may take place at the level of the free bases, the nucleosides, or at the nucleotide stage. The first alternative has been treated in Chapter 23. Here, the interconversion of the nucleosides and the nucleotides will be discussed. The following exemplifying equations are formulated with the nucleosides; in some instances they occur with the nucleotides. The reactions to be considered are:

$$\text{Inosine} \rightleftharpoons \text{Adenosine} \tag{14}$$

$$\text{Inosine} \rightleftharpoons \text{Xanthosine} \rightleftharpoons \text{Guanosine} \tag{15}$$

$$\text{Orotidine} \rightleftharpoons \text{Uridine} \tag{16}$$

The same conversions may occur with the corresponding deoxyribose nucleosides. In addition, some of the reactions appear to be restricted to the deoxyribonucleosides, as follows:

$$\text{Deoxycytidine} \rightleftharpoons \text{5-Methyldeoxycytidine} \tag{17}$$

$$\text{Deoxyuridine} \rightleftharpoons \text{Thymidine} \tag{18}$$

The type of cells determines which of these reactions occur and whether they or transglycosidations are the preferred routes of synthesis. Not all of

[43a] J. L. Ott and C. H. Werkman, *Arch. Biochem. and Biophys.* **48**, 483 (1954).

[44] G. A. Kritskiĭ, *Doklady Akad. Nauk S.S.S.R.* **70**, 667 (1950), cf. *Chem. Abstr.* **44**, 7367c (1950); *Doklady Akad. Nauk S.S.S.R.* **82**, 289 (1950), cf. *Chem. Abstr.* **46**, 7600f (1952).

the mechanisms (14) to (18) have been studied in detail. In some instances the reversibility has not yet been demonstrated.

a. Inosine⇌ Adenosine

Adenosine deaminase is the best studied of these enzymes.[3,45-47] The specificity is usually high in the tissue enzyme which attacks only adenosine and deoxyadenosine;[47,48] no other related nucleosides with substituents in the purine or carbohydrate are attacked,[16] nor is free adenine deaminated. In *L. helveticus* a deaminase is found which catalyzes the deamination of adenine riboside but not of adenine deoxyriboside. This is in contrast to a number of other bacteria, such as *E. coli* and *L. casei*, which have a deaminase resembling that from tissues.[43] Relatively low specificity is shown by the adenosine deaminase from *Aspergillus oryzae*.[49] Kaplan *et al.*[50] found that the purified enzyme deaminates adenosine, 5-adenylic acid, 3-adenylic acid, diphosphopyridine nucleotide, adenosine diphosphate, adenosine triphosphate, and the fragment of DPN consisting of adenosine diphosphate and ribose (DPN minus nicotinamide). Deoxyadenosine was not tested, and adenine, triphosphopyridine nucleotide, and 2-adenylic acid were not deaminated.

b. 5-Adenylic Acid Deaminase

Another deaminase, which is specific for 5-adenylic acid, was discovered by Schmidt[46] in muscle tissue. It attacks 5-deoxyadenylic acid but not 3-adenylic acid.[6,51] More recent investigations of this enzyme have resulted in confirmation and extension of Schmidt's data.[52,53] This deaminase occurs in voluntary muscle, nerve tissue, auricular muscle of the heart, and in erythrocytes; it is lacking in kidney, liver, intestine, and smooth muscle.[54]

c. Amination of Inosine and Inosinic Acid

The deaminations caused by adenosine deaminase and by 5-adenylic acid deaminase go to completion, and no reversal with an excess of ammonia has been observed. Glutamine has been assumed by many authors to be the donor of the amino group. Suggestive evidence for this hypothesis has been

[45] P. György and H. Röthler, *Biochem. Z.* **187,** 194 (1927).
[46] G. Schmidt, *Z. physiol. Chem.* **179,** 243 (1928).
[47] T. Brady, *Biochem. J.* **36,** 478 (1942).
[48] H. M. Kalckar, *J. Biol. Chem.* **167,** 445 (1947).
[49] H. K. Mitchell and W. D. McElroy, *Arch. Biochem.* **10,** 351 (1946).
[50] N. O. Kaplan, S. P. Colowick, and M. M. Ciotti, *J. Biol. Chem.* **194,** 579 (1952).
[51] C. E. Carter, *J. Am. Chem. Soc.* **73,** 1537 (1951).
[52] G. D. Lu and D. M. Needham, *Biochem. J.* **35,** 392 (1941).
[53] H. B. Stoner and H. N. Green, *Biochem. J.* **39,** 474 (1945).
[54] E. J. Conway and R. Cooke, *Biochem. J.* **33,** 479 (1939).

furnished by Kalckar and Rittenberg.[55] After administration of ammonium citrate, labeled with N^{15}, they found a high concentration of the isotope in the 6-amino nitrogen of the adenylic acid of skeletal muscle. This indicated a rapid reversible deamination. Under the same conditions the amino nitrogen of the glutamic acid of the muscle proteins showed an isotope concentration which was only one-fifth of that of the 6-amino nitrogen of the 5-adenylic acid, while the amide nitrogen had a higher isotope value. The acid amide group of glutamine (and perhaps asparagine) may be the immediate precursor of the amino group. Weil-Malherbe[56] has studied this system with washed or dialyzed brain homogenates and believes that the amination occurs at the expense of high-energy phosphate bonds. Elliott and Gale have studied this system with *Staphylococcus aureus*.[57]

d. Guanylic Acid Deaminase

Guanylic acid deaminase was discovered by Schmidt;[58] it seems to be different from guanase, but further characterization is needed. Guanylic acid formation may occur by amination of xanthylic acid, deamination of 2,6-diaminopurine nucleotide,[59,60] or by incorporation of an N-C unit instead of formate into 4-amino-5-imidazolecarboxamide. These alternatives are hypothetical; on the other hand, the occurrence of guanase in many cells and the reactivity of nucleoside phosphorylase with guanine in presence of the pentose esters suggest that guanine may be the precursor of the guanine nucleosides.

Glutamine may be suspected as a donor of the amino group as in the case of adenine nucleotides, but no experiments are recorded. Deamination of guanosine always seems to be attended by splitting into base and carbohydrate.[58] The biosynthesis of guanylic acid has barely been touched by investigators.[60a]

e. Cytidine Deamination

Deamination of cytidine has been observed in liver,[58,60b] in blood,[61] in *E. coli*, and in yeast.[30] According to Wang *et al.*,[62] the action of the enzyme from *E. coli* and from yeast extends to cytosine riboside and deoxyriboside, while

[55] H. M. Kalckar and D. Rittenberg, *J. Biol. Chem.* **170,** 455 (1947).
[56] H. Weil-Malherbe, *Biochem. J.* **54,** vi (1953).
[57] W. H. Elliott and E. F. Gale, *Nature* **161,** 129 (1948).
[58] G. Schmidt, *Z. physiol. Chem.* **208,** 185 (1932).
[59] A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.* **193,** 481 (1951).
[60] G. P. Wheeler and H. E. Skipper, *Federation Proc.* **12,** 289 (1953).
[60a] B. Magasanik and M. S. Brooke, *J. Biol. Chem.* **206,** 83 (1954).
[60b] L. Grossman and D. W. Visser, *J. Biol. Chem.* **209,** 447 (1954).
[61] E. J. Conway and R. Cooke, *Biochem. J.* **33,** 457 (1939).
[62] T. P. Wang, H. Z. Sable and J. O. Lampen, *J. Biol. Chem.* **184,** 17 (1950).

Klein[6] suggests separate enzymes in tissues for the two cytosine nucleosides. No cytidylic acid deaminase free from phosphatase has been obtained. Deamination of cytosine by a different enzyme from some microorganisms has been observed; it does not act on cytidine or cytidylic acid, but can deaminate 5-methylcytosine to thymine.[63] As yet nothing is known about the deamination of 5-methylcytosine deoxyriboside, nor is there any information about reversal of these deaminations.

f. Decarboxylation of Orotic Acid and Its Homologues

The activity of orotic acid as a precursor of pyrimidines is apparent from the results of numerous experiments on the incorporation of the labeled acid and the appearance of the isotope in the nucleic acid pyrimidines.[37] All attempts to decarboxylate orotic acid *in vitro* have failed, however, and it appears that removal of the carboxyl group takes place on the nucleoside or nucleotide level. This assumption is supported by the recent isolation of orotidine[32] and by the observations of Hurlbert and Potter.[64,65] They found that injection of orotic acid-6-C^{14} into rats leads to accumulation of labeled uridine-5′-phosphate and diphosphate in the liver. *In vitro* experiments should soon clarify the steps of orotic acid utilization.

g. Formation of 5-Methyldeoxycytidine and Thymidine

It is not known whether 5-methyldeoxycytidine and thymidine are formed by methylation of deoxycytidine and deoxyuridine; thymidine could also result from deamination of 5-methyldeoxycytidine. The methyl group may be introduced into the bases or even precursors of the latter. There are no experiments with isolated enzyme systems. *In vivo* studies have shown that the methyl group originates from one-carbon units such as formate.[66,67] Folic acid appears to play a part in the transfer of the single carbon unit. Shive believe that a direct synthesis of a conjugated form of thymine is possible.[68]

III. Biosynthesis of Nucleotides

Information on the biosynthesis of nucleosides is far from complete; our knowledge of nucleotide formation is scanty at best. A tempting speculation is the assumption of enzymic phosphorylation in position 3 or 5 of the pentose of nucleosides. On the other hand, observations have been recorded which are contrary to this hypothesis. According to these recent reports,

[63] E. Chargaff and J. Kream, *J. Biol. Chem.* **175,** 993 (1948).
[64] R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.* **195,** 267 (1952).
[65] R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.* **209,** 1 (1954).
[66] D. Elwyn and D. B. Sprinson, *J. Am. Chem. Soc.* **72,** 3317 (1950).
[67] J. R. Totter, E. Volkin, and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1521 (1951).
[68] W. Shive, *Federation Proc.* **12,** 639 (1953).

the assembling of nucleotides may begin with the production of pentose-phosphate and its combination with simple nitrogenous compounds, leading to aliphatic ribotides.[69,70,70a] The nitrogenous system is finished afterwards by addition of the necessary structure, followed by ring closure (see Chapter 23). According to this concept, nucleosides and free bases play no part in anabolism; they are products of catabolism only as indicated in the following scheme:

$$\begin{array}{l} \text{Purine precursors} \rightarrow \text{Precursor riboside} \rightarrow \text{Precursor ribotide} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \uparrow\downarrow \\ \text{(Uric acid, etc.)} \leftarrow \text{Purine} \rightleftharpoons \text{Purine riboside} \rightleftharpoons \text{Purine ribotide} \end{array} \tag{19}$$

Inosinic acid appears to be the key intermediate; adenylic acid and guanylic acid may be formed from it by the reactions discussed in Section II.8. Opponents of this concept emphasize that nucleoside phosphorylase and adenosine phosphokinase action provide a plausible route of nucleotide synthesis with adenylic acid as a primary product. In both instances, experiments have been restricted so far to the purine ribose compounds. A decision in favor of one of these alternatives or the assumption of multiple pathways would be premature at this time in view of the limited experimental data which are available.

1. Experiments with Labeled Nucleosides and Nucleotides

In the formulation of an acceptable metabolic scheme, one may look for guidance from the results of tracer experiments. Most of this work has been done with labeled bases, and, among other results, it has brought to light the strict separation of purine and pyrimidine metabolism.

Experiments with labeled nucleosides are not so numerous, because these compounds are more difficult to prepare; biosynthesis of them is usually employed.[71-73] Parenteral administration to animals and incorporation into microbiological growth media have shown that they are incorporated into nucleic acids. In rats the purine nucleosides were less effectively incorporated than adenine,[74] but the reverse is true for pyrimidine nucleosides compared with free pyrimidines. Table I shows some data of Lowy *et al.*,[75] which indicate that the label of adenosine is found in both constituent

[69] J. M. Buchanan and M. P. Schulman, *J. Biol. Chem.* **202,** 241 (1953).

[70] G. R. Greenberg, *Federation Proc.* **12,** 651 (1953).

[70a] D. A. Goldthwait and R. A. Peabody, *Federation Proc.* **13,** 218 (1954).

[71] F. J. DiCarlo, A. S. Schultz, P. M. Roll, and G. B. Brown, *J. Biol. Chem.* **180,** 329 (1949).

[72] E. Hammarsten, P. Reichard, and E. Saluste, *J. Biol. Chem.* **183,** 105 (1950).

[73] I. A. Rose and B. S. Schweigert, *J. Biol. Chem.* **202,** 635, (1953).

[74] P. M. Roll, B. G. Brown, F. J. DiCarlo, and A. S. Schultz, *J. Biol. Chem.* **180,** 333 (1949).

[75] B. A. Lowy, J. Davoll, and G. B. Brown, *J. Biol. Chem.* **197,** 591 (1952).

TABLE I

PER CENT RENEWAL OF NUCLEIC ACID PURINES IN THE RAT FROM PURINE NUCLEOSIDES[75]

Compound examined	Compound administered[a]				
	Adenosine	Inosine	Diamino-purine riboside	Guanosine	Crotonoside
PNA:					
Adenine	2.4	0.67	0.04	0.01	0.05
Guanine	0.61	0.28	1.2	0.27	0.30
DNA:					
Mixed Purines	0.05	0.07	0.07	0.03	0.02

[a] Nucleosides administered at 0.2 mM. per kg. of body weight per day.

TABLE II

PER CENT RENEWAL OF NUCLEIC ACID PYRIMIDINES IN THE RAT FROM PYRIMIDINE NUCLEOSIDES AND NUCLEOTIDES[37, 72]

Compound examined	Compound administered[a]			
	Cytidylic acid	Cytidine	Uridylic acid	Uridine
PNA:				
Cytosine	10.6	7.2	0.85	0.47
Uracil	5.2	5.9	1.21	0.42
DNA:				
Cytosine	4.3	4.4	0.46	0.49
Thymine	1.4	1.6	0.52	0.34

[a] Nucleotides administered at 0.4 mM. per kg. of body weight per day, nucleosides at 0.34 mM. per kg per day.

ribonucleic acid purines; inosine is inferior to adenosine; the isotope of 2,6-diaminopurine riboside, guanosine, and crotonoside recurs only in guanosine. No significant incorporation of the isotope into deoxyribonucleic acid was observed. Table II shows the results of experiments with pyrimidine nucleosides[72] and nucleotides.[37] The label of each compound is found preferentially in the corresponding base, but there is also extensive conversion into the other pyrimidines. Reichard and Estborn[76] have carried out similar experiments with labeled deoxynucleosides. In rats the label of deoxycytidine was found in cytosine and thymine of deoxynucleic acid, but not in the pyrimidines of ribonucleic acid. Thymidine was recovered only in the thymine of the deoxynucleic acid. This indicates that the methyla-

[76] P. Reichard and B. Estborn, *J. Biol. Chem.* **188,** 839 (1951).

tion of the pyrimidine leading to thymine or one of its homologues is irreversible. Rose and Schweigert[73] studied the utilization of totally labeled cytidine-C^{14} by the rat. It recurs in both the ribo- and deoxyribonucleic acid, and the incorporation does not involve prior cleavage of the glycosidic linkage; this favors the concept that a mechanism exists for the conversion of ribose into deoxyribose at the nucleoside or nucleotide level.

Similar studies have been carried out with a variety of microorganisms such as yeast and bacterial species.[73,77,78] *L. casei* utilized purine ribosides, but not as effectively as free purines or purine nucleotides.[37] The fragments of uniformly labeled ribonucleic acid, prepared by biosynthesis using the phytoflagellate *Euglena gracilis*, were tested with *E. coli* and *L. leichmannii*.[73] Cytosine and cytidine were essentially equivalent as sources of ribonucleic acid pyrimidines, but the ribose of totally labeled cytidine was incorporated less effectively than the base.

The inferiority of purine ribosides,[77] compared with the nucleotides[78] and the free purine bases, to serve as nucleic acid precursors has been interpreted to indicate that they cannot be intermediates in the conversion of free purines into nucleic acid purines. On the other hand, the possibility of a primary conversion of nucleosides into the free bases has been excluded.[76,77] One may assume that the purines react with ribose-1,5-diphosphate and that this reaction is faster than the addition of phosphate to preformed nucleosides. A different picture obtains with pyrimidines. The failure of the bases other than orotic acid[79] to be incorporated, and the precursor activity of the nucleosides, suggest a role of the latter as intermediates of nucleic acid formation.

Observation of overall reactions in cells and tissues by means of tracer techniques fails to reveal detail. On the other hand, *in vitro* studies have yielded only fragmentary information which is not yet sufficient to map a metabolic scheme. Reactions which may figure prominently in nucleotide synthesis will be discussed below.

2. Enzymic Reactions of Nucleotide Metabolism

a. 5-Nucleotidase and 3-Nucleotidase

Phosphatases usually have a wide range of specificity, but the nucleotidases are an exception. Evidence of the existence of a specific 5-nucleo-

[77] M. E. Balis, D. H. Levin, G. B. Brown, G. B. Elion, H. Vander-Werff, and G. H. Hitchings, *J. Biol. Chem.* **199**, 227 (1952).

[78] M. E. Balis, D. H. Levin, G. B. Brown, G. B. Elion, H. Vander-Werff, and G. H. Hitchings, *J. Biol. Chem.* **200**, 1 (1953).

[79] H. Arvidson, N. A. Eliasson, E. Hammarsten, P. Reichard, H. V. Ubisch, and S. Bergstrom, *J. Biol. Chem.* **179**, 169 (1949).

tidase was accumulated by Reis,[80] Gulland and Jackson,[81] and Mann.[82] A fiftyfold purification and separation from other phosphatases has recently been accomplished by Heppel and Hilmoe,[83] who used bull seminal plasma as a source material. The enzyme has a pH optimum at 8.2 and shows specificity toward the following ribose-5-phosphate derivatives, with decreasing activity in the order given: cytidine-5′-phosphate, uridine-5′-phosphate, inosine-5′-phosphate, adenosine-5′-phosphate, and, to a lesser degree, ribose-5-phosphate. No splitting with numerous other phosphate esters including adenosine-2′- and 3′-phosphate, guanylic, uridylic, and cytidylic acids from yeast was observed. The enzyme was also found in snake venoms and in potato extracts.[81,83,83a,b]

The significance of 5-nucleotidase in nucleotide metabolism is suggested by its specificity. About its role in synthetic processes as little can be stated as about other hydrolytic enzymes. Moreover, we do not know whether 5-nucleotides or 3-nucleotides are the primary products employed by nature in polynucleotide synthesis.

Shuster andKaplan[84] have found a 3-nucleotidase in germinating barley. They separated it from 5-nucleotidase and nonspecific phosphatases and observed that 3-adenylic, 3-guanylic, and 3-inosinic acids are split rapidly. 3-Uridylic acid and 3-cytidylic acid are split more slowly.

b. Adenosine Phosphokinase

Ostern and co-workers[85] have suggested an interconversion of adenosine 3′-phosphate and adenosine-5′-phosphate with adenosine as an intermediate. They were concerned mainly with the origin of coenzyme adenylic acid, which they assumed to arise from ribonucleic acid via adenosine. Adenosine phosphates were found to accumulate, if adenosine was incorporated into fermenting yeast macerates; ribonuclease and phosphatase action were assumed to provide the adenosine in cells from polynucleotides. However, it is not certain that ribonucleic acid supplies coenzyme moieties; the reverse could be assumed as well. Ostern's experiments were carried out before tracer techniques were available. Recent results make it probable that coenzyme and polynucleotide synthesis are not intimately related.

[80] J. Reis, *Bull. soc. chim. biol.* **16,** 7, 385 (1934); **22,** 36 (1940); *Enzymologia* **2,** 183 (1937); **5,** 25 (1938).

[81] J. M. Gulland and E. M. Jackson, *Biochem. J.* **32,** 597 (1938).

[82] T. Mann, *Biochem. J.* **39,** 451 (1945).

[83] L. A. Heppel and R. J. Hilmoe, *J. Biol. Chem.* **188,** 665 (1951).

[83a] F. G. Fischer and H. Dörfel, *Z. physiol. Chem.* **296,** 232 (1954).

[83b] M. Hartmann and W. Bosshard, *Helv. Chim. Acta* **21,** 1554 (1938).

[84] L. Shuster and N. O. Kaplan, *J. Biol. Chem.* **201,** 535 (1953).

[85] P. Ostern and J. Terszakowec, *Z. physiol. Chem.* **250,** 155 (1937); P. Ostern, T. Baranowski, and J. Terszakowec, *ibid.* **251,** 258 (1938); P. Ostern, J. Terszakowec, and S. Hubl, *ibid.* **255,** 104 (1938).

The phosphorylation of adenosine by a yeast enzyme in presence of a suitable phosphate donor was confirmed by Sable.[86] He also reported that D-ribose is phosphorylated while deoxyribose and other pentoses were inert in this yeast system. Ribokinase and adenosine phosphokinase are not identical, and these enzymes could not be detected in tissues. The action of yeast and tissue adenosine phosphokinase was studied in more detail by Caputto,[87] and by Kornberg and Pricer[59] who established the equation

$$\text{Adenosine} + \text{ATP} \rightleftharpoons \text{adenosine-5'-phosphate} + \text{ADP} \qquad (20)$$

The pH optimum is approximately 6, and Mg^{++} or Mn^{++} is required. There is specificity for adenosine with the exception of 2-aminoadenosine (2,6-diamino-9-β-D-furanosylpurine), which proved reactive but inferior to adenosine. 2-Oxyadenosine (crotonoside), deoxyadenosine, inosine, guanosine, uridine, cytidine, and several synthetic nucleosides proved inert.

The failure of various tissue enzymes to phosphorylate nucleosides is disappointing.[86,88-90] Phosphorylated pentoses are secured in the cell by other routes, if a direct phosphorylation is not feasible (see Chapter 22). In view of the failure to achieve experimentally the enzymic phosphorylation of nucleosides, one may be inclined to assume combination of phosphorylated pentoses with bases or base fragments. Experiments along this line are described below, but they are far from satisfactory.

c. Nucleotide-N-ribosidase

The early work with nucleolytic enzymes seemed to indicate that the phosphoric acid group in the nucleotides has to be removed before the glycosidic linkage can be resolved by enzymes.[5,91] This concept has been challenged by Ishikawa and Komita.[92] Some experiments of G. Schmidt[58] seemed to indicate that guanylic acid, when deaminated, is split simultaneously to yield xanthine and ribose phosphate; the formation of the latter was inferred from the observation that no inorganic phosphate appeared during the incubation. Small amounts of what seemed to be ribose phosphate were isolated, but the phosphate content was found to be 20% below the theoretical value. Moreover, the behavior of the product during acid hydrolysis resembled that of ribose-5-phosphate rather than ribose-3-phosphate. The Japanese investigators[92,93] made similar observations with

[86] H. Z. Sable, *Proc. Soc. Exptl. Biol. Med.* **75**, 215 (1950).
[87] R. Caputto, *J. Biol. Chem.* **189**, 801 (1951).
[88] G. E. Youngburg, *Arch. Biochem.* **4**, 137 (1944).
[89] F. Schlenk and M. J. Waldvogel, *Arch. Biochem.* **9**, 455 (1946), **12**, 181 (1947).
[90] A. Canzanelli, R. Guild, and D. Rapport, *Am. J. Physiol.* **162**, 168 (1950).
[91] P. A. Levene and A. Dmochowski, *J. Biol. Chem.* **93**, 563 (1931).
[92] H. Ishikawa and Y. Komita, *J. Biochem.* (*Japan*) **23**, 351 (1936).
[93] Y. Komita, *J. Biochem.* (*Japan*) **25**, 405 (1937); **23**, 191 (1938).

crude tissue enzymes. Their assumption of a glycosidic split without prior removal of the phosphoric acid group (nucleotide-*N*-ribosidase action) is based on the observation of a constant level of inorganic phosphate during incubation, while according to the conventional scheme with nucleotidase action as the first step there should be liberation of phosphate. However, crude tissue preparations and long incubation times were used and the carbohydrate phosphoric acid ester was not characterized.[93]

In the light of more recent observations, another interpretation of these experiments is possible.[94] Guanylic acid may be split first by phosphatase (eq. 21). The resulting guanosine is cleaved by phosphorolysis (eq. 22), and the resulting ribose-1-phosphate is rearranged by mutase action to ribose-5-phosphate (eq. 23) and to hexose-6-monophosphate[89,95] and other esters (see Chapter 22).

$$\text{Guanosine-3'-phosphate} \rightarrow \text{guanosine} + \text{phosphate} \tag{21}$$

$$\text{Guanosine} + \text{phosphate} \rightarrow \text{guanine} + \text{ribose-1-phosphate} \tag{22}$$

$$\text{Ribose-1-phosphate} \rightarrow \text{ribose-5-phosphate} \rightarrow \text{hexose-6-phosphate} \tag{23}$$

No phosphate appears in the reaction medium under these circumstances, provided reactions (22) and (23) are faster than (21). Proof of the existence of 3-nucleotide-*N*-ribosidase is lacking, at present; it would require the isolation and characterization of ribose-3-phosphate.

Other experiments in this category are those of Wajzer.[96,97] He reports that the phosphorolysis of inosine by liver enzymes is attended by a secondary reaction leading to inosinic acid. Another set of his experiments deals with the reaction of ribose-3-phosphate with hypoxanthine, adenine, and guanine. The analytical techniques, however, are inadequate and the results are partly hypothetical.

It is tempting to assume that nucleotides are synthesized by the interaction of bases or base precursors with ribose-1,5-diphosphate. The prospect[98] of securing adequate amounts of this ester heralds important developments.

d. Enzymic Phosphorylation of Nucleosides by Phosphate Transfer

Brawerman and Chargaff[99] have made important observations on the formation of nucleotides by phosphate transfer; this process is catalyzed by a phosphatase obtained from a commercial preparation of malt diastase. In the presence of sodium phenyl phosphate as phosphate donor it converts ribose and deoxyribose nucleosides into the nucleotides. Inorganic phos-

[94] F. Schlenk, *Advances in Enzymol.* **9,** 455 (1949).
[95] M. J. Waldvogel and F. Schlenk, *Arch. Biochem.* **22,** 185 (1949).
[96] J. Wajzer, *Arch. sci. physiol.* **1,** 493 (1947).
[97] J. Wajzer and F. Baron, *Bull. soc. chim. biol.* **31,** 750 (1949).
[98] H. Klenow and B. Larsen, *Arch. Biochem. and Biophys.* **37,** 488 (1952).
[99] G. Brawerman and E. Chargaff, *J. Am. Chem. Soc.* **75,** 2020 (1953).

phate cannot be an intermediate in this reaction, because it is inhibitory. Many nucleotides are obtained from the requisite nucleosides by prolonged incubation; and much inorganic phosphate is formed by concomitant splitting of the phenylphosphate. The phosphorylation seems to occur in position 5 of the sugar; the reaction products were identified by chromatography and could be dephosphorylated by 5-nucleotidase.[99]

It appears that one is dealing here with a model system; the artificial phosphate donor, phenyl phosphate, under natural conditions may be replaced by a biological phosphoric acid ester, perhaps a nucleotide such as adenosine or inosine phosphate. In recent years several similar phosphate transfer mechanisms by hydrolytic enzymes have been observed.[100] The analogy of this phosphate transfer to the transglycosidation described by McNutt[40] is obvious. Important results may be expected from the exploration of this field.

IV. Biosynthesis by Reaction of Pentose or Pentosephosphate with Incomplete Pyrimidine and Purine Systems

The first suggestion that pentose or pentose phosphate may combine with a nitrogenous precursor of nucleic acid bases came from the studies of Loring and Pierce[101] and Mitchell and co-workers.[102,103] These investigators used various mutants of *Neurospora crassa*; some of them were found to utilize the free pyrimidines very poorly in comparison with the correspoding nucleosides, an observation which has its counterpart in tissue and whole animal studies which likewise proved inert toward the pyrimidines with the exception of orotic acid.[37] This suggested that the pyrimidine bases are not intermediates in nucleoside synthesis as exemplified by Fig. 6.

Experiments to verify this hypothesis have so far remained suggestive rather than conclusive. For lack of synthetic aliphatic ribosides, numerous surmised precursors of orotic acid have been tested with various organisms[102,104] (see also Chapter 23). If any of them were the primary reactant with the pentose, it should surpass orotic acid in its growth-promoting ability or in the rate of incorporation into nucleic acids. Such precursors have not been found.[104,105,105a]

The question remains whether ring closure to orotic acid, or formation of

[100] R. K. Morton, *Nature* **172,** 65 (1953).
[101] H. S. Loring and J. G. Pierce, *J. Biol. Chem.* **153,** 61 (1944).
[102] H. K. Mitchell and M. B. Houlahan, *Federation Proc.* **6,** 506 (1947).
[103] H. K. Mitchell, M. B. Houlahan, and J. F. Nyc, *J. Biol. Chem.* **172,** 525 (1948).
[104] L. D. Wright, K. A. Valentik, D. S. Spicer, J. W. Huff, and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.* **75,** 293 (1950).
[105] U. Lagerkvist, P. Reichard, and G. Ehrensvärd, *Acta Chem. Scand.* **5,** 1212 (1951).
[105a] L. D. Wright, C. S. Miller, and C. A. Driscoll, *Proc. Soc. Exptl. Biol. Med.* **86,** 215 (1954).

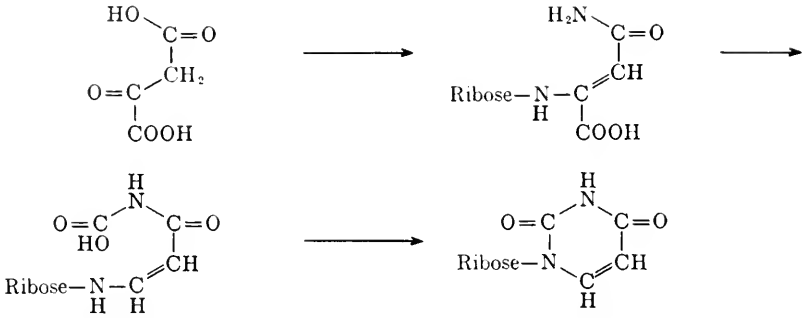
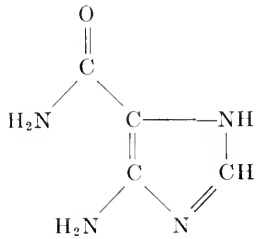


FIG. 6. A suggested mechanism for the biosynthesis of uridine.[102]

the glycosidic bond by combination with the pentose, occurs first. The former reaction has not been tested *in vitro* with the nucleosidase presently available, and no enzyme has been found so far which is capable of combining the 1-phosphate esters of ribose or deoxyribose with orotic acid. The isolation of orotidine by Mitchell and his co-workers[32] will facilitate the study of this problem. In Chapter 23 it has been pointed out that orotic acid, labeled in many different ways, is readily incorporated into the nucleic acid fraction of various types of cells. This favors strongly the assumption that orotic acid is a key intermediate in pyrimidine nucleoside and nucleotide formation.

On much firmer ground is the claim of Greenberg[106,107] that purine nucleosides and nucleotides may be formed by combination of the carbohydrate with purine precursors, followed by completion of the purine system. Experiments of this type have been restricted to inosine and inosinic acid. We owe the most significant contributions to Greenberg and to Buchanan and their co-workers; rapid progress may be anticipated. Figuring most prominently as a purine precursor, in these studies, is 4-amino-5-imidazolecarboxamide:[108]



This incomplete hypoxanthine was first encountered in sulfadiazine-inhib-

[106] G. R. Greenberg, *Federation Proc.* **9,** 179 (1950).
[107] G. R. Greenberg, *J. Biol. Chem.* **190,** 611 (1951).
[108] A. Windaus and W. Langenbeck, *Ber.* **56,** 683 (1923).

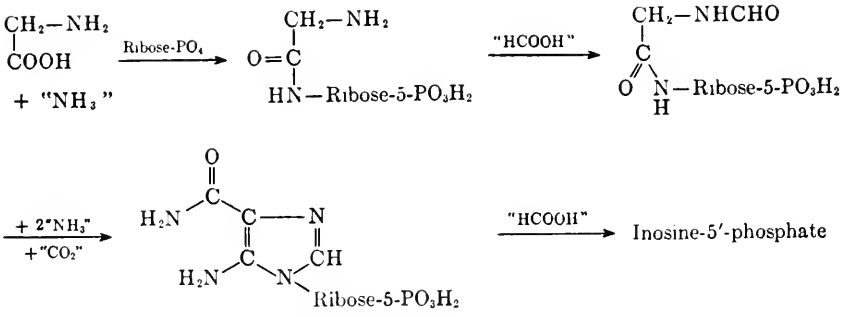


FIG. 7. Working scheme of nucleotide biosynthesis.[70]

ited cultures of *E. coli* by Stetten and Fox;[109] it was identified by Shive and his associates.[110,111] Greenberg[107] came to the conclusion that the carboxamide as such is not an intermediate in the formation of inosinic acid, nor does inosine have to be considered as an obligatory precursor. There is indication that the carboxamide ribotide is the immediate precursor of inosinic acid. Schulman and Buchanan studied the incorporation of formate-C^{14} into this compound, which yields carbon atom 2 of the base.[112] In presence of inosinic acid a high rate of exchange of the carbon atom 2 of the purine with the carbon of labeled formate was found. Hypoxanthine and inosine were inert.[112]

The concept of Greenberg[107] that the free purines may not be the products of direct assembly of their structural units, but instead may be formed by combination of aliphatic nitrogenous compounds with ribose phosphate, can be formulated as in Fig. 7.[70,70a] There is some indication that the purine nucleus is assembled to the carboxamide stage while in linkage with pentose, because Greenberg[113] was able to show that the carboxamide riboside rather than the carboxamide itself is produced by young cultures of sulfadiazine-inhibited *E. coli*. Earlier investigators missed this compound because their conditions of isolation inadvertently caused hydrolysis. On the other hand, instead of being an intermediate, the carboxamide riboside and carboxamide itself may be merely split products of inosinic acid.[113] Buchanan and co-workers[17,17a] observed that purified beef liver nucleosidase is capable of combining the carboxamide with ribose-1-phosphate. The carboxamide riboside is readily converted to the ribotide by liver or yeast enzymes in presence of ATP and Mg^{++}.[114]

[109] M. R. Stetten and C. L. Fox, Jr., *J. Biol. Chem.* **161**, 333 (1945).
[110] W. Shive, W. W. Ackermann, M. Gordon, M. E. Getzendanner, and R. E. Eakin, *J. Am. Chem. Soc.* **68**, 725 (1947).
[111] J. M. Ravel, R. E. Eakin, and W. Shive, *J. Biol. Chem.* **172**, 67 (1948).
[112] M. P. Schulman and J. M. Buchanan, *J. Biol. Chem.* **196**, 513 (1952).
[113] G. R. Greenberg, *J. Am. Chem. Soc.* **74**, 6307 (1952).
[114] G. R. Greenberg, *Federation Proc.* **12**, 211 (1953).

The importance of the reactions outlined above for purine synthesis via inosinic acid is discussed in Chapter 23, and the role of the *citrovorum* factor is described on pages 335 and 336. Many details of inosinic acid synthesis will have to be elaborated.[115] It remains to be seen whether this is the main path of nucleotide synthesis or merely an alternative. As yet, there is little information about other nucleotides[116,117] and there are no data on a comparable system synthesizing deoxyribose nucleotides. The only indication pointing in this direction is the tentative identification of carboxamide deoxyriboside in cultures of *E. coli*.[118]

V. Role of B-Vitamins in Nucleoside and Nucleotide Synthesis

A function of several B-vitamins in nucleic acid metabolism has been observed. Some of them act as coenzymes in the synthesis of the pentoses and in the production of amino acid and other purine and pyrimidine precursors. This discussion will be restricted to vitamin cofactors participating in the metabolism of the nucleosides, nucleotides, and closely related compounds. From the preceding part of this chapter it is apparent that our knowledge concerning the reaction pattern of these compounds is still very limited; it is not surprising, therefore, that little can be reported about the cofactors of these enzyme reactions.

1. Vitamin B_{12}

The relationship of vitamin B_{12} to nucleoside and nucleic acid synthesis was recognized in 1948.[38, 119,120] It was found that deoxyribose nucleosides, particularly thymidine, can replace vitamin B_{12} in promoting growth of some *Lactobacilli*.[121,122] Another effect was observed with *L. leichmannii*; the phosphate incorporation into the deoxyribonucleic acid of this organism was greatly increased, as measured by the use of radioactive phosphate.[123] Thymine cannot usually replace thymidine as a substitute for vitaminB_{12}, which points to a role in deoxyribose formation or in the attachment of the carbohydrate to the base.[124] Contrary to expectations, however, the vitamin has not been found in nucleosidase nor in the enzymes synthesizing deoxyribose.[125] The site of action may be in the nucleoside transglycosidase.

[115] E. D. Korn and J. M. Buchanan, *Federation Proc.* **12,** 233 (1953).
[116] W. H. Marsh, *J. Biol. Chem.* **190,** 633 (1951).
[117] W. J. Williams and J. M. Buchanan, *J. Biol. Chem.* **202,** 253 (1953).
[118] R. Ben-Ishai, E. D. Bergmann, and B. E. Volcani, *Nature* **168,** 1124 (1951).
[119] W. Shive, J. M. Ravel, and R. E. Eakin, *J. Am. Chem. Soc.* **70,** 2614 (1948).
[120] L. D. Wright, H. R. Skeggs, and J. W. Huff, *J. Biol. Chem.* **175,** 475 (1948).
[121] E. Hoff-Jørgensen, *J. Biol. Chem.* **178,** 525 (1949).
[122] W. Shive, *Ann. N. Y. Acad. Sci.* **52,** 1212 (1950).
[123] I. Z. Roberts, K. B. Roberts, and P. H. Abelson, *J. Bacteriol.* **58,** 709 (1949).
[124] T. H. Jukes and E. L. R. Stokstad, *Vitamins and Hormones* **9,** 1 (1951)
[125] E. Racker, *J. Biol. Chem.* **196,** 347 (1952).

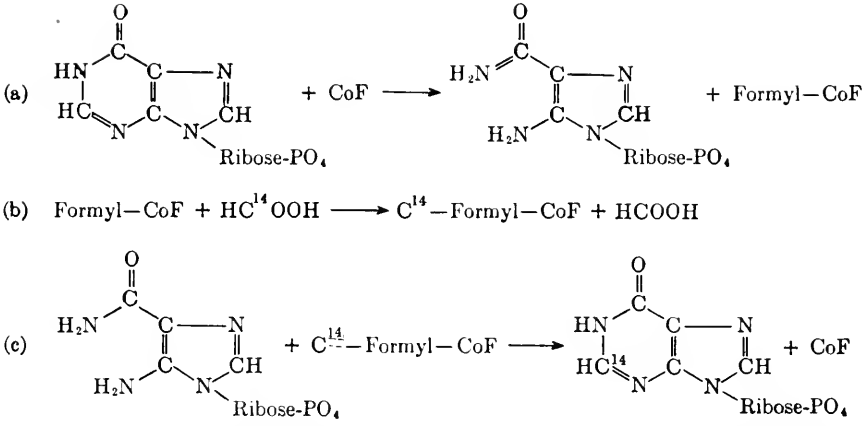


FIG. 8. Possible reactions of formate and inosinic acid.[69] CoF (coenzyme F) indicates 5,6,7,8-tetrahydrofolic acid. Formyl-CoF (citrovorum factor) indicates N-5-formyl-5,6,7,8-tetrahydrofolic acid. (See also Fig. 6 in Chapter 23.)

2. CITROVORUM FACTOR (LEUCOVORIN, FOLINIC ACID SF, COENZYME F)

Citrovorum factor is named for its growth-promoting activity for *Leuconostoc citrovorum*. It is a derivative of folic acid, namely, *N*-5-formyl-5,6,7,8-tetrahydropteroylglutamic acid.[126,127] A role of the citrovorum factor in purine synthesis was suggested by Shive,[122] and his hypothesis was put to a test by Buchanan and Schulman[69] in the system of inosinic acid formation from 4-amino-5-imidazolecarboxamide ribotide. Both folic acid and citrovorum factor were found to activate the incorporation of radioactive formate into inosinic acid by exchange with carbon atom 2 of the base in presence of pigeon liver extracts. Citrovorum factor surpassed folic acid greatly in its activating effect, but the amounts used (5 to 45 μg./ml.) are rather high.[127a] Three- to fourfold increase in the rate of formyl incorporation was achieved. Buchanan and Schulman[69] represent the reaction as shown in Fig. 8.

Besides carbon atom 2, purine carbon 8 and the 5-methyl group of thymine may be derived from formic acid[67,128,128a,b] and a similar formulation as given above appears possible. Other effects may concern one-carbon transfers in the synthesis of aliphatic purine and pyrimidine precursors, but these

[126] A. Pohland, E. H. Flynn, R. A. Jones, and W. Shive, *J. Am. Chem. Soc.* **73,** 3247 (1951).

[127] H. P. Broquist, M. J. Fahrenbach, J. A. Brockman, Jr., E. L. R. Stokstad, and T. H. Jukes, *J. Am. Chem. Soc.* **73,** 3535 (1951).

[127a] G. R. Greenberg, *Federation Proc.* **13,** 221 (1954).

[128] J. M. Buchanan, J. C. Sonne, and A. M. Delluva, *J. Biol. Chem.* **173,** 81 (1948).

[128a] D. A. Goldthwait and A. Bendich, *J. Biol. Chem.* **196,** 841 (1952).

[128b] P. Berg, *J. Biol. Chem.* **205,** 145 (1954).

reactions are remote from the ring assembly by several steps; hence their discussion will be omitted.

3. *p*-Aminobenzoic Acid and Folic Acid

An obvious function of *p*-aminobenzoic acid and folic acid in purine and pyrimidine metabolism is to serve as part of the structure of citrovorum factor. There are numerous experimental results pointing to this relationship; in most instances fastidious bacteria or organisms with an antagonist-induced deficiency have been used, but no *in vitro* observations permitting exact formulations have been made. Since numerous reviews on this subject are available, a discussion will be omitted.[68,129-131]

VI. Addendum

Nucleoside-5′-triphosphates

The most significant recent development is the isolation of various ribonucleoside-5′-triphosphates. These compounds may occupy a key position in future concepts of polynucleotide synthesis. Guanosine-5′-triphosphate and uridine-5′-triphosphate were obtained from rabbit muscle;[133] the concentration was 2 to 4 % of that of the ATP fraction. Yeast appears to be a good source of uridine-5′-triphosphate.[134] All of the ribonucleoside triphosphates were found in tumor and other tissues.[135,136] The occurrence of deoxyadenosine diphosphate and triphosphate in muscle and kidney has been suggested.[137]

The studies of Kalckar and his co-workers[138,139] have revealed important information about the function and biogenesis of ribonucleotide triphosphates.[139a,b] The following enzymic reactions were observed:

$$\text{Uridine triphosphate} + \text{adenosine diphosphate} \rightleftharpoons \text{uridine diphosphate} + \text{adenosine triphosphate} \quad (24)$$

[129] W. Shive, *Vitamins and Hormones* **9,** 76 (1951)

[130] T. H. Jukes, *Federation Proc.* **12,** 633 (1953).

[131] J. R. Totter, *J. Cellular Comp. Physiol.* **41,** Suppl. 1, 241 (1953).

[133] R. Bergkvist and A. Deutsch, *Acta Chem. Scand.* **7,** 1307 (1953).

[134] S. H. Lipton, S. A. Morell, A. Frieden, and R. M. Bock, *J. Am. Chem. Soc.* **75,** 5449 (1953).

[135] R. B. Hurlbert, H. Schmitz, A. F. Brumm, and V. R. Potter, *J. Biol. Chem.* **209,** 23 (1954); H. Schmitz, R. B. Hurlbert, and V. R. Potter, *ibid.* **209,** 41 (1954); L. Hecht, V. R. Potter, and E. Herbert, *Biochim. et Biophys. Acta* **15,** 134 (1954).

[136] J. Sacks, L. Lutwak, and P. D. Hurley, *J. Am. Chem. Soc.* **76,** 424 (1954).

[137] H. Z. Sable, P. B. Wilber, A. E. Cohen, and M. R. Kane, *Biochim. et Biophys Acta* **13,** 156 (1954).

[138] H. M. Kalckar, *Biochim. et Biophys. Acta* **12,** 250 (1953).

[139] A. Munch-Petersen, H. M. Kalckar, E. Cutolo, and E. E. B. Smith, *Nature* **172,** 1036 (1953); H. M. Kalckar, *Science* **119,** 479 (1954).

[139a] I. Lieberman, A. Kornberg, and E. S. Simms, *J. Am. Chem. Soc.***76,** 3608 (1954).

[139b] J. L. Strominger, L. A. Heppel, and E. S. Maxwell, *Arch. Biochem. and Biophys.* **52,** 488 (1954).

Such a phosphate shift may be of general importance as indicated by the discovery of the reaction:[140]

$$\text{Adenosine triphosphate} + \text{inosine diphosphate} \rightleftharpoons \text{adenosine diphosphate} + \text{inosine triphosphate} \quad (25)$$

A connection of uridine triphosphate with co-waldenase is indicated by the reaction:[138,139]

$$\text{Uridine diphosphate glucose (galactose)} + \text{pyrophosphate} \rightleftharpoons \text{uridine triphosphate} + \alpha\text{-glucose-1-phosphate } (\alpha\text{-galactose-1-phosphate}) \quad (26)$$

Nucleoside Metabolism

A hydrolytic ribonucleosidase has been obtained from fish muscle by Tarr.[141] After 650-fold purification it was found to act on purine ribosides and cytidine, but not on uridine. Continued studies by Friedkin[142] on pyrimidine deoxyriboside phosphorylase from calf kidney have shown complete lack of reactivity of orotic acid and 5-methylcytosine with deoxyribose-1-phosphate. However, in addition to the bases specified in equations (8) and (9) (p. 318), some substituted synthetic pyrimidines showed moderate activity.[142a]

5-Hydroxymethylcytosine

The discovery of 5-hydroxymethylcytosine (Chapter 10) (16 to 17 moles per 100 moles of DNA bases) in some bacteriophages (*E. coli*, T_2, T_4, and T_6, but not in T_5 and T_7) poses interesting questions.[142b] The host cell does not contain this base. It is not clear yet at what level (base, nucleoside, or nucleotide) the conversion of host pyrimidine units into 5-hydroxymethylcytosine occurs.[143,144,144a]

Thymidine and Other Pyrimidine Compounds

Sprinson and co-workers[145] have extended their work on the formation of the methyl group of thymine or thymidine. Experiments with 2,3-deuterio-3-C^{14}-N^{15}-serine have led them to believe that formate is not an intermediate, because both hydrogens accompany the β-carbon in the trans-

[140] P. Berg and W. K. Joklik, *Nature* **172,** 1008 (1953); *J. Biol. Chem.* **210,** 657 (1954).
[141] H. L. A. Tarr, *Federation Proc.* **13,** 309 (1954).
[142] M. Friedkin and D. Roberts, *J. Biol. Chem.* **207,** 257 (1954).
[142a] F. Weygand, A. Wacker, and H. Dellweg, *Z. Naturforsch.* **7b,** 19 (1952); D. B. Dunn, J. D. Smith, S. Zamenhof, and G. Griboff, *Nature* **174,** 305 (1954).
[142b] R. L. Sinsheimer, *Science* **120,** 551 (1954).
[143] G. R. Wyatt and S. S. Cohen, *Nature* **170,** 1072 (1952); *Biochem. J.* **55,** 774 (1953).
[144] L. L. Weed and T. A. Courtenay, *J. Biol. Chem.* **206,** 735 (1954).
[144a] S. S. Cohen and L. L. Weed, *J. Biol. Chem.* **209,** 789 (1954).
[145] D. Elwyn, A. Weissbach, and D. B. Sprinson, *J. Am. Chem. Soc.* **73,** 5509 (1951); D. Elwyn and D. B. Sprinson, *J. Biol. Chem.* **207,** 467 (1954).

fer. Friedkin and co-workers[146] have observed rapid utilization of thymidine-C^{14} for DNA synthesis in embryonated hens' eggs. Thymine-C^{14} was not utilized and no appreciable activity could be found in the ribonucleic acid fraction.

Lieberman and Kornberg[147] consider the possibility that dihydroorotic acid rather than orotic acid may be the precursor of nucleic acid pyrimidines (see Chapter 23, Addendum).

Nucleosides and Nucleotides as Nucleic Acid Precursors

The following recent reports on utilization of labeled compounds augment the metabolic picture: Roll and Weinfeld[148] have obtained adenylic and guanylic acids *a* and *b* which were uniformly labeled with C^{14}, N^{15}, and P^{32}. These were injected into rats; after one day, the nucleic acid fragments were isolated. There was no specific utilization of the phosphorus of any of the administered nucleotides. Ribose of the adenylic acids was incorporated to about 80 %, which strongly suggests that the major pathway of utilization does not involve a rupture of the ribosidic linkage. In contrast hereto, the incorporation of the ribose of the guanylic acids was only 20 per cent of that of the base.

It would seem that dephosphorylation of adenylic acid under these conditions does not militate against its role as an intermediate of polynucleotide synthesis. Loss of the phosphoric acid group may be incidental during transport to the tissues; it may facilitate penetration into the cells.

New data on pyrimidine nucleotide, phosphate-P^{32}, and orotic acid incorporation have appeared.[149,150] An extensive examination of pyrimidine-deficient mutants produced by ultraviolet irradiation of *Aerobacter aerogenes* has been reported by Nelson and Shapiro.[151] No mutants were found which responded to pyrimidine nucleosides or nucleotides only, but not to the bases. On the other hand, nucleosides and nucleotides always served equally well as did the bases.

Further work on 4-amino-5-imidazolecarboxamide has confirmed the occurrence of its riboside and ribotide.[152] Involvement of thymidine in the utilization of this base is suggested.[153]

[146] M. Friedkin, D. Tilson, and D. Roberts, *Federation Proc.* **13,** 214 (1954).
[147] I. Lieberman and A. Kornberg, *Biochim. et Biophys. Acta* **12,** 223 (1953).
[148] P. M. Roll and H. Weinfeld, *Federation Proc.* **13,** 282 (1954); P. M. Roll, H. Weinfeld, and G. B. Brown, *Biochim. et Biophys. Acta* **13,** 141 (1954).
[149] L. L. Weed and D. W. Wilson, *J. Biol. Chem.* **202,** 745 (1953).
[150] K. Moldave and C. Heidelberger, *J. Am. Chem. Soc.* **76,** 679 (1954).
[151] E. V. Nelson and S. K. Shapiro, *J. Bacteriol.* **67,** 692 (1954).
[152] J. S. Gots, *Nature* **172,** 256 (1953).
[153] J. M. Weaver and W. Shive, *J. Am. Chem. Soc.* **75,** 4628 (1953).

Phosphate Transfer Enzymes

The phosphate transfer enzymes, first reported in germinating barley, have now been found also in human prostate and in rat liver.[154] The barley and liver enzymes produce only the 5-nucleotides whereas the prostate enzyme converts the nucleosides into all possible mononucleotide isomers. Besides phenyl phosphate, 5-nucleotides can serve as phosphate donors with the barley and liver enzyme.

Phosphoribokinase and Nucleotide Phosphorylase

Kalckar and his co-workers have presented new information on 5-adenylic acid formation.[138,155] Pigeon liver enzyme contains 5-phosphoribokinase which catalyzes the following reaction:

$$\text{Ribose-5-phosphate} + \text{ATP} \rightleftharpoons \text{ribose-1,5-diphosphate} + \text{ADP} \qquad (27)$$

Another enzyme from the same source, termed nucleotide phosphorylase, brings about the reaction:

$$\text{Ribose-1,5-diphosphate} + \text{adenine} \rightleftharpoons \text{5-adenylic acid} + \text{phosphate} \qquad (28)$$

[154] G. Brawerman and E. Chargaff, *J. Am. Chem. Soc.* **75,** 4113 (1953).
[155] M. Saffran and E. Scarano, *Nature* **172,** 949 (1953).

CHAPTER 25

Biosynthesis of Nucleic Acids

GEORGE BOSWORTH BROWN AND PAUL M. ROLL

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I. Introduction

In 1877, Miescher[1] proposed that in the egg the nucleic acid of the embryo is formed by alteration of the protein vitellin. This was undoubtedly the first published speculation on the mode of biosynthesis of nucleic acids, and, although many theories concerning the origin of these compounds have subsequently been presented, we still cannot adequately describe the processes by which nucleic acids are formed in the living cell. This is partly due to the complex nature of the nucleic acids, and to the incompletely understood heterogeneity of the substances. It is also apparent that the PNA and DNA of one species are different from those of other species, that within a single species there is a multiplicity of PNA's differing from tissue to tissue, and that there are within each cell several metabolically distinct PNA's. Although the concept of a constancy of composition of DNA within a species is prevalent, it has recently been shown that the DNA from a single organ can be separated into at least two fractions. Therefore investigations of the biosynthesis of nucleic acids have involved studies on mixtures of compounds, and it is not impossible that the same pathway of synthesis is not common to all of the nucleic acids.

1. Nonessentiality of Nucleic Acid Constituents in Mammalian Nutrition

Because of the universal distribution of nucleic acids and their apparent importance to cellular processes, it is not surprising that organisms are able to accomplish the biosynthesis of nucleic acids from simple and generally available metabolites. Except for a few microorganisms, this is true. Numerous investigations have clearly demonstrated that both growing and adult animals are capable of synthesizing nucleic acids and are not dependent upon any external source for the purines, pyrimidines, or sugars of these crucial compounds. In 1874, Miescher pointed out[2] that the ability of the migrating salmon to form large amounts of nucleic acid for spermatozoa indirectly indicates the power to synthesize these compounds from other body constituents. The earliest direct experimental demonstrations were

[1] F. Miescher, "Die Histochemischen und Physiologischen Arbeiten," p. 108. F. C. W. Vogel, Leipsig, 1897.

[2] F. Miescher, *Hoppe-Seyler's Med.-Chem. Unters.* **6**, 138 (1874).

those of Tichomiroff in 1885[3] on the increase of purines in developing silkworm ova and of Kossel in 1886,[4] who, although he could find no purines in fresh hens' eggs was able to isolate purines from the nucleoproteins of the chick embryo after several days' incubation. Experiments involving the use of purine-free diets by Socin[5] in 1891 with growing mice, and by McCollum[6] in 1909 with growing rats, demonstrated that in these animals, where nucleic acid synthesis was certainly occurring, no exogenous purines were required. Other experiments by Osborne and Mendel,[7] by Benedict,[8] and by Ackroyd and Hopkins[9] have demonstrated this same fact, although the latter authors erroneously concluded that purines arise from histidine and arginine. A result of the fact that, over the last two decades, purified and fully characterized diets have been available for nutritional studies has been the repeated demonstration that none of the organic constituents of the nucleic acids is necessary in the diet.

II. The Nature of the Substances Which Can Be Utilized as Polynucleotide Precursors

Although classical nutritional and balance studies lead to the conclusion that nucleic acids can be synthesized from simple substances, they give no indication as to the materials used or the mechanisms involved in nucleic acid biosynthesis. It was not until isotope tracer techniques became available that nucleic acid precursors could be identified and that pathways of nucleic acid biosynthesis could begin to be described. The earliest experiments on nucleic acids in which isotopes were used were those of Hevesy, beginning in 1940,[10] in which it was shown that when inorganic phosphate labeled with P^{32} was administered to animals it was rapidly incorporated into the nucleic acids. Radioactive phosphorus has proved to be a popular isotope in nucleic acid research and has been particularly useful in the demonstration of biological differences among several nucleic acid fractions based on differences in their renewals. It has also been used as an indicator to demonstrate the effects of various agents upon the processes of nucleic acid synthesis.

The pioneering experiments of Schoenheimer and his group were extended to the nucleic acids when ammonium citrate labeled with heavy nitrogen

[3] A. Tichomiroff, *Z. physiol. Chem.* **9,** 518 (1885).
[4] A. Kossel, *Z. physiol. Chem.* **10,** 248 (1886).
[5] C. A. Socin, *Z. physiol. Chem.* **15,** 93 (1891).
[6] E. V. McCollum, *Am. J. Physiol.* **25,** 120 (1909).
[7] T. B. Osborne and L. B. Mendel, *Z. physiol. Chem.* **80,** 307 (1912).
[8] S. R. Benedict, *J. Lab. Clin. Med.* **2,** 1 (1916–17).
[9] H. Ackroyd and F. G. Hopkins, *Biochem. J.* **10,** 551 (1916).
[10] G. Hevesy, "Radioactive Indicators." Interscience, New York, 1948.

(N^{15}) was used[11] to show that ammonia is a precursor of the nitrogen of the nucleic acid purines and pyrimidines in the rat and the pigeon. Other investigations involving the use of labeled compounds have demonstrated that many simple substances can be utilized in the biosynthesis of various polynucleotide components (Chapters 22, 23), and extensive use has been made of the incorporation of such smaller precursors into polynucleotides in the course of studies directed at the nucleic acids themselves.

1. Precursors of the Polynucleotide Purines

The demonstration of the participation of simple substances in the biosynthesis of purines and pyrimidines, and the failure to find a nutritional requirement for any nucleic acid derivative (other than phosphate) for most living forms, cannot be interpreted to mean that purines, pyrimidines, nucleosides, or nucleotides are not involved in the biosynthesis of nucleic acids. Indeed, it does not seem likely that during the process of cellular biosynthesis of the nucleic acids all of the simple precursors are simultaneously assembled and "snapped together" into a complex polynucleotide. Rather, it would seem that simple precursors would be built into complex compounds and these then constructed into more complex substances until finally the polynucleotide is formed. On the assumption that this is the pattern of nucleic acid biosynthesis, a considerable amount of work has been done to determine the possible nature of the intermediates involved in such a scheme. The search for these intermediates has centered chiefly upon the hydrolysis products of nucleic acid, not only because such compounds might reasonably be expected to be precursors, but also because they could be made available as labeled compounds either by organic synthesis, biosynthesis, or by enzymic synthesis.

a. Adenine and Derivatives

The first demonstration of the participation of a nucleic acid constituent in a process of biosynthesis was the finding[12,13] that, in the rat, dietary adenine is incorporated into tissue nucleic acids as adenine and that it is also a precursor of nucleic acid guanine. When adenine-1,3-N_2^{15} was fed at a level of 0.2 mM. per kilogram of body weight per day for 3 days, 5.4% of the adenine and 3.2% of the guanine of the mixed nucleic acids of the viscera were derived from the dietary compound, and these values were increased to 13.7 and 8.2%, respectively, when the level of administration was increased to 1.5 mM. per kilogram of body weight per day. These values represent incorporations into mixed PNA and DNA of rat viscera, but, in

[11] F. W. Barnes, Jr., and R. Schoenheimer, *J. Biol. Chem.* **151,** 123 (1943).

[12] G. B. Brown, P. M. Roll, and A. A. Plentl, *Federation Proc.* **6,** 517 (1947).

[13] G. B. Brown, P. M. Roll, A. A. Plentl, and L. F. Cavalieri, *J. Biol. Chem.* **172,** 469 (1948).

an experiment in which the PNA and DNA were separated, little incorporation of adenine into DNA was detected.[14] However it was later demonstrated that adenine is utilized for DNA synthesis in rapidly growing tissues, for example, intestine[15,16] and regenerating liver.[17] In all species subsequently tested, adenine has proved to be moderately to extensively incorporated into the polynucleotides, and in most instances it is also converted into polynucleotide guanine.

In an experiment in which yeast nucleic acid uniformly labeled with N^{15} [18] was hydrolyzed by alkali, presumably to a mixture of mononucleotides, and this was injected into rats at a level of 0.4 mM. per kilogram of body weight per day, it was found that only 3.7 % of both adenine and guanine of the visceral nucleic acids had been derived from the labeled compounds.[20] Thus it is evident that free adenine is a much better precursor of nucleic acids than is combined adenine. This was further demonstrated in an experiment by Lowy *et al.*[21] in which adenosine-8-C^{14} [22] was administered to rats at the 0.2-mM. level and was found to be incorporated into PNA and DNA adenine about only one-half as well as was adenine.

Adenylic acid uniformly labeled with N^{15} was isolated from labeled yeast nucleic acid,[18] and when injected into rats at twice the level at which adenosine was given was found[24] to be incorporated to only twice the extent, which indicated that it too was a less effective precursor than was adenine. This was later confirmed in experiments in which the 2′- and 3′-isomers[25] of adenylic acid-8-C^{14} were administered[23] separately to rats at the same level as was adenosine. It was found that the adenylic acids were incorporated to approximately the same extent as was adenosine and also that each isomer

[14] G. B. Brown, M. L. Petermann, and S. S. Furst, *J. Biol. Chem.* **174,** 1043 (1948).
[15] R. Abrams, *Arch. Biochem. and Biophys.* **33,** 436 (1951).
[16] D. A. Goldthwait and A. Bendich, *J. Biol. Chem.* **196,** 841 (1952).
[17] S. S. Furst, P. M. Roll, and G. B. Brown, *J. Biol. Chem.* **183,** 251 (1950).
[18] The nucleic acid was isolated from yeast which had been grown in a medium containing $(N^{15}H_4)_2SO_4$.[19]
[19] F. J. DiCarlo, A. S. Schultz, P. M. Roll, and G. B. Brown, *J. Biol. Chem.* **180,** 329 (1949).
[20] P. M. Roll, G. B. Brown, F. J. DiCarlo, and A. S. Schultz, *J. Biol. Chem.* **180,** 333 (1949).
[21] B. A. Lowy, J. Davoll, and G. B. Brown, *J. Biol. Chem.* **197,** 591 (1952).
[22] Synthesized via a labeled purine and chloroacetoribofuranose.[23]
[23] J. Davoll and B. A. Lowy, *J. Am. Chem. Soc.* **74,** 1563 (1952).
[24] P. M. Roll and I. Weliky, *Federation Proc.* **10,** 238 (1951).
[25] Yeast was grown in a medium containing adenine-8-C^{14} [26] and, from the nucleic acid, purine nucleotides were isolated by ion-exchange procedures.[27]
[26] S. E. Kerr, K. Seraidarian, and G. B. Brown, *J. Biol. Chem.* **188,** 207 (1951).
[27] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 1471 (1950).
[28] G. B. Brown, P. M. Roll, and H. Weinfeld, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 385. Johns Hopkins Press, Baltimore, 1951.

was utilized to the same extent. However, adenosine-5′-phosphate-8-C^{14} [29] was incorporated to an appreciably smaller extent than were those three compounds.[31] In all of the experiments involving adenosine and adenylic acid, it was found that there was a conversion of the adenine moiety into PNA guanine but that the guanine was always labeled to less than one-half the extent of PNA adenine. There was at best only a small incorporation of these compounds into DNA. On the basis of these results alone, the possibility of the incorporation of adenosine and adenylic acids into the nucleic acids of the rat via a prior degradation to adenine cannot be excluded.

The only other organisms in which the incorporation of adenosine and adenylic acids have been studied are yeast and *Lactobacillus casei*. In yeast, only a very small incorporation of any of the nucleosides or nucleotides was observed.[26] On the other hand, in *L. casei* there was a considerable utilization of these derivatives, and results with adenosine[32] parallel those obtained with the rat in that the nucleoside is not utilized as well as is the free base. However in *L. casei* adenosine-2′-phosphate is utilized scarcely at all while adenosine-3′-phosphate is incorporated as well as is adenine.[33]

b. Guanine and Derivatives

In 1944, Plentl and Schoenheimer[34] reported that dietary guanine was not utilized by the rat for nucleic acid synthesis, and this result was confirmed[12,13] in parallel with the experiments which revealed the incorporation of adenine-1,3-N_2^{15}. However, in both experiments guanine-2-*amino*-1,3-N_3^{15} was administered so that, during the catabolism of the purine to allantoin (a process which was known to occur from the presence of highly labeled allantoin in the urine), the 2-amino group contributed $N^{15}H_3$ to the body pool ammonia. This resulted in a small generalized labeling of all purines and pyrimidines, and consequently a trace incorporation of guanine was undetectable in the experiments. Subsequently, by the use of the more sensitive tracer carbon-14, Balis *et al.*[35] were able to show that guanine-8-C^{14} was incorporated to about the extent of 0.1 % in the nucleic acid guanine of the rat. Abrams[15] found that guanine-2-C^{14} was incorporated to a somewhat greater extent into the guanine of the PNA of rat intestine, and was

[29] Prepared by phosphorylation of synthetic adenosine-8-C^{14} by the use of adenosine phosphokinase.[30]

[30] A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.* **193,** 481 (1951).

[31] H. Weinfeld and P. M. Roll, *Federation Proc.* **12,** 287 (1953).

[32] M. E. Balis, D. H. Levin, G. B. Brown, G. B. Elion, H. VanderWerff, and G. H. Hitchings, *J. Biol. Chem.* **199,** 227 (1952).

[33] M. E. Balis, D. H. Levin, G. B. Brown, G. B. Elion, H. VanderWerff, and G. H. Hitchings, *J. Biol. Chem.* **200,** 1 (1953).

[34] A. A. Plentl and R. Schoenheimer, *J. Biol. Chem.* **153,** 203 (1944)

[35] M. E. Balis, D. H. Marrian, and G. B. Brown, *J. Am. Chem. Soc.* **73,** 3319 (1951).

able to detect a very slight conversion of guanine to PNA adenine. In the black mouse, a small but significant utilization of guanine-2-*amino*-1,3-N_3^{15} was detected.[36]

Hammarsten and Reichard[37] investigated the incorporation of guanosine uniformly labeled with N^{15} [38] and concluded that it was not utilized for nucleic acid synthesis by the rat. However, by the use of guanosine-8-C^{14},[22] a slight but definite incorporation (0.3 %) was found[21] into the guanine of the rat visceral PNA, with no significant conversion of guanosine to PNA adenine. Yet the experiment[20] in which mixed N^{15} nucleotides were injected into rats had indicated that this mixture had contained a quite effective guanine precursor. An explanation of that latter result became apparent when it was found[24] that guanylic acid-N^{15} was well incorporated into the guanine of the PNA of rat viscera and may have also been used to a slight extent for the formation of DNA guanine.

It was found that in *L. casei*[40] the incorporation of guanine and its derivatives is as extensive as that of adenine and its derivatives. Guanine is well incorporated into PNA, guanosine[32] is a poorer precursor, and guanosine-3′-phosphate is well utilized whereas guanosine-2′-phosphate is not.[33]

c. Other Purines and Derivatives

In an effort to determine the nature of the intermediates involved in the conversion of adenine to guanine, several nonnucleic acid purines have been investigated as possible nucleic acid precursors. Isoguanine,[41] hypoxanthine, xanthine,[42] and uric acid[43] (labeled with N^{15}) were each fed to rats and were found to be ineffective as nucleic acid precursors. It should be noted that the studies with hypoxanthine, xanthine, and isoguanine in the rat involved N^{15}-labeled samples and that trace incorporations such as that subsequently detected for guanine are not excluded. However, 2,6-diaminopurine[44,45] is incorporated into polynucleotide guanine in the rat; in one experiment as much as 4 % of the guanine was found to have been derived from the 2,6-diaminopurine but no conversion into polynucleotide adenine

[35] G. B. Brown, A. Bendich, P. M. Roll, and K. Sugiura, *Proc. Soc. Exptl. Biol. Med.* **72,** 501 (1949).

[37] E. Hammarsten and P. Reichard, *Acta Chem. Scand.* **4,** 711 (1950).

[38] Prepared from *Escherichia coli* grown with $N^{15}H_3$.[37,39]

[39] P. Reichard and B. Estborn, *J. Biol. Chem.* **188,** 839 (1951).

[40] M. E. Balis, D. H. Levin, G. B. Brown, G. B. Elion, H. VanderWerff, and G. H. Hitchings, *J. Biol. Chem.* **196,** 729 (1952).

[41] A. Bendich, G. B. Brown, F. S. Philips, and J. B. Thiersch, *J. Biol. Chem.* **183,** 267 (1950).

[42] H. Getler, P. M. Roll, J. F. Tinker, and G. B. Brown, *J. Biol. Chem.* **178,** 259 (1949).

[43] G. B. Brown, P. M. Roll, and L. F. Cavalieri, *J. Biol. Chem.* **171,** 835 (1947).

[44] A. Bendich and G. B. Brown, *J. Biol. Chem.* **176,** 1471 (1948).

[45] A. Bendich, S. S. Furst, and G. B. Brown, *J. Biol. Chem.* **185,** 423 (1950).

was detected. The 4-amino-5-imidazolecarboxamide is also well utilized for polynucleotide purine synthesis by the rat.[46]

Lowy *et al.*[21] investigated the incorporation of the nucleosides of several of these purines. 2,6-Diaminopurine riboside was incorporated into polynucleotide guanine with only a trace conversion to polynucleotide adenine observed. The 2,6-diaminopurine riboside was utilized only about one-third as extensively as was the free purine. Crotonoside (isoguanine riboside) was incorporated to the same small extent as was guanosine. Inosine was utilized for both adenine and guanine synthesis but only about one-third as readily as was adenosine.

d. The Maintenance of the Integrity of the Purine Skeleton

None of the evidence available has indicated a significant metabolic lability of individual positions of the skeleton of intact purines.

The conversion in the rat of adenine-1,3-N_2^{15} into guanine with the isotope in the guanidine portion first suggested[12,13] that that conversion was accomplished with the retention of the intact purine ring. 2,6-Diaminopurine-2-*amino*-1,3-N_3^{15} showed a similar behavior, and diaminopurine-2-C^{13} also led[45] to guanine with the bulk of the C^{13} found in the guanidine derived from the 2-position of the guanine. A discussion[47] of the possible metabolic lability of position 2 of diaminopurine involved the assumptions that a small discrepancy in the C^{13} values was experimentally significant, and that the over twofold difference in the incorporations of N^{15} and C^{13} diaminopurine in different groups of rats was also significant. This latter difference between individuals is, however, less than the threefold difference which was observed[48] for the incorporation of adenine in different individuals maintained under nominally identical experimental conditions.

The essential integrity of the purine ring is also demonstrated by the parallel incorporation (with the C^{14} always slightly lower) of the isotopes of adenine-1,3-N_2^{15}-8-C^{14} into each of the adenylic and guanylic acid isomers in the rat,[48] by the identical incorporation and conversion into adenine of guanine-2-C^{14} or of guanine-8-C^{14} in *L. leichmanii*,[49] and by the nonincorporation of $N^{15}H_3$ of the medium into the ring nitrogens of exogenous purines in *Escherichia coli*.[50] In a variety of experiments in *L. casei* no metabolic lability of either the 2- or 8-positions of the purines was indicated.[40] Specific investigations using formate in the pigeon and the chick[51] and in folic acid-deficient rats,[52] and glycine in the mouse,[53] did not indicate any unusual lability of individual positions of the purine ring. The precision of most of the experimental evidence leaves something to be desired, but the deviations are not sufficient to suggest that there is any appreciable lability of the ring.

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[46] C. S. Miller, S. Gurin, and D. W. Wilson, *Science* **112,** 654 (1950).

[47] M. Gordon, *Science* **114,** 110 (1951).

[48] D. H. Marrian, V. L. Spicer, M. E. Balis, and G. B. Brown, *J. Biol. Chem.* **189,** 533 (1951).

[49] F. Weygand, *Abstr. 2nd Intern. Congr. Biochem., Paris* p. 96 (1952).

[50] A. L. Koch, F. W. Putnam, and E. A. Evans, Jr., *J. Biol. Chem.* **197,** 105, 113 (1952).

[51] W. H. Marsh, *J. Biol. Chem.* **190,** 633 (1951).

[52] G. R. Drysdale, G. W. E. Plaut, and H. A. Lardy, *J. Biol. Chem.* **193,** 533 (1951).

[53] C. Heidelberger and G. A. LePage, *Proc. Soc. Exptl. Biol. Med.* **76,** 464 (1951).

When adenine-6-C^{14} and $N^{15}H_3$ were administered to *E. coli*,[50] there was little (6%) exchange of the amino group of the adenine incorporated, while 60% of the amino group of the guanine was derived from the $N^{15}H_3$. This experiment also indicated no lability of carbon 6.

In this connection it might be noted that, in the catabolism[12,13] of adenine-1,3-N_2^{15} or[43] of uric acid-1,3-N_2^{15} to uniformly labeled allantoin via the symmetrical intermediate, hydroxyacetylene diureide carboxylic acid, it is the 6-carbon which is lost. There does not now appear to be any connection between the symmetrical distribution of the ureido moieties in the *in vivo* oxidation of uric acid and the equal labeling of carbons 2 and 8 of purines by 1-carbon precursors.

2. Precursors of Polynucleotide Pyrimidines

a. Pyrimidines

The pattern of utilization of pyrimidines and pyrimidine derivatives differs markedly from the general scheme of utilization of the purines and their derivatives. Uracil, thymine,[34] cytosine,[54] and the unnatural 2,6-diaminopyrimidine[55] were all found to be ineffective as nucleic acid precursors in the rat. Later, a small incorporation of thymine-1,3-N_2^{15} into the polynucleotide thymine of regenerating rat liver was detected,[28] and a very small incorporation of uracil-2-C^{14} into rat PNA is reported.[56] The detection of an incorporation of thymine in regenerating liver is reminiscent of the far greater incorporation of adenine into regenerating as compared to normal liver DNA.

The only pyrimidine that can be readily utilized as a precursor of nucleic acid in the rat is orotic acid (uracil-4-carboxylic acid). Although this compound occurs naturally (Chapter 3), it is not a nucleic acid component and yet all of the polynucleotide pyrimidines of the rat can be extensively derived from orotic acid-1,3-N_2^{15}.[57] It has also been found that the polynucleotide pyrimidines can be derived from orotic acid-2-C^{14} in rat and human tissues,[58,59] from orotic acid-4-C^{14} in rats,[58] and from orotic acid-6-C^{14} in rats[60] and in yeast.[61,62] All this evidence suggests that orotic acid is transformed into polynucleotide pyrimidines without undergoing extensive degradation. This question is discussed further in Chapter 23.

[54] A. Bendich, H. Getler, and G. B. Brown, *J. Biol. Chem.* **177,** 565 (1949).

[55] A. Bendich, W. D. Geren, and G. B. Brown, *J. Biol. Chem.* **185,** 435 (1950).

[56] R. J. Rutman, A. Cantarow, K. E. Paschkiss, and B. Allanoff, *Science* **117,** 282 (1953).

[57] H. Arvidson, N. A. Eliasson, E. Hammarsten, P. Reichard, H. Ubisch, and S. Bergström, *J. Biol. Chem.* **179,** 169 (1949).

[58] L. L. Weed and D. W. Wilson, *J. Biol. Chem.* **189,** 435 (1951).

[59] L. L. Weed, *Cancer Research* **11,** 470 (1951).

[60] R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.* **195,** 257 (1952).

[61] M. Edmonds, A. M. Delluva, and D. W. Wilson, *J. Biol. Chem.* **197,** 251 (1952).

[62] The alternative numbering systems possible (cf. Chapter 3) may account for the references to both orotic acid-6 and 4-C^{14} from this laboratory.

b. Pyrimidine Derivatives

Although *free* nucleic acid pyrimidines are not well utilized for nucleic acid synthesis, it has been found that injection into rats of an alkaline hydrolysate of N^{15}-labeled nucleic acid[18] resulted in extensive labeling of polynucleotide pyrimidines, which indicated that *combined* pyrimidines can be utilized for nucleic acid biosynthesis.[20] Studies of the metabolism in the rat of cytidine and uridine[63] labeled with N^{15} (Hammarsten *et al.*[64]) and of cytidylic and uridylic acids[28] also labeled with N^{15} have been made. It is fortunate that almost identical experimental conditions in the two laboratories allow a reasonable comparison of the results (Table I). The pyrimidines of both PNA and DNA are derived extensively from both cytidine and cytidylic acid and the nucleoside and nucleotide are equally effective as precursors. The incorporations, too, are of the magnitude of those observed with adenine. In both types of nucleic acid there is a preferential incorporation of both precursors into cytosine but with considerable transformation into the other pyrimidine. Uridine and uridylic acid are less effective precursors than the corresponding cytosine derivatives, but again it is seen that these compounds are incorporated into the pyrimidines of both PNA and DNA. Uridylic acid is a better precursor of PNA pyrimidines than is uridine but the two compounds are equally well incorporated into DNA pyrimidines.

It has been pointed out[64] that since these free pyrimidines cannot be utilized these results indicate that a transformation of ribose to deoxyribose occurs without rupture of the ribosidic linkage. This hypothesis is validated by studies[65] on the incorporation into rat nucleic acid of cytidine randomly labeled with C^{14} in both[66] the pyrimidine and the sugar. When cytidine, uridine, deoxycytidine, and thymidine were isolated from the rat polynucleotides, it was found that in each nucleoside the base and the sugar were approximately equally labeled, indicating that the injected cytidine had been incorporated as a unit and that the conversion of ribose to deoxyribose had occurred with the ribosidic linkage intact.

c. Specific Precursors of the DNA Bases

Several N^{15}-labeled deoxyribonucleosides[38] have been investigated as possible nucleic acid precursors in the rat.[39] Hypoxanthine deoxyriboside proved to ineffective. Deoxycytidine was found to be a precursor of cytosine and thymine of DNA but was not at all utilized for PNA synthesis. Thymidine was utilized for the synthesis of polynucleotide thymine only.

[63] Prepared from yeast grown with $N^{15}H_3$.[64]

[64] E. Hammarsten, P. Reichard, and E. Saluste, *J. Biol. Chem.* **183,** 105 (1950).

[65] I. A. Rose and B. S. Schweigert, *J. Biol. Chem.* **202,** 635 (1953).

[66] Prepared from *Euglena gracilis* grown in the presence of $C^{14}O_2$.[65]

TABLE I

PER CENT RENEWAL OF PYRIMIDINES IN THE POLYNUCLEOTIDES OF THE VISCERA IN THE RAT, FROM PYRIMIDINE NUCLEOSIDES AND NUCLEOTIDES[a]

	Compound administered			
	Cytidine	Cytidylic acid	Uridine	Uridylic acid
PNA:				
Cytosine	7.2	10.6	0.47	0.85
Uracil	5.9	5.2	0.42	1.21
DNA:				
Cytosine	4.4	4.3	0.49	0.46
Thymine	1.6	1.4	0.34	0.52

[a] Nucleosides administered at 0.34 mM. per kg. body wt. per day.[64] Nucleotides administered at 0.4 mM. per kg. body wt. per day.[28]

These nucleosides and possibly thymine and thymine-4-carboxylic acid[28] are unique in that they are the only compounds that have been found to be specific DNA precursors.

3. A Summary of Compounds Used as Polynucleotide Precursors in the Rat

The exploratory metabolic experiments with a newly prepared labeled compound have almost always involved studies of only the nucleic acids from the pooled viscera of a minimum number of animals. In several cases there are also variations in the size of the administered dose and of times and route of administration, and these facts make detailed comparisons of the degrees of incorporation impractical. In the investigations cited here, analyses of other nucleic acid components, proteins, urinary excretion products, etc., furnished evidence that the compounds are specific precursors of the moieties in question. The evidence has all indicated that purines or their derivatives are not precursors of the pyrimidines. Nor are pyrimidines or their derivatives precursors of purines, although[64] with cytidine-N^{15} there was some appearance of the nitrogen in the DNA purines, a result which was not obtained[28] with cytidylic acid-N^{15}. The renewals of the 2′- and 3′-isomers of the purine nucleotides obtainable from PNA have been found to be essentially the same whether the precursor be phosphate,[67] formate[68,69] adenine,[48] nucleosides,[21] or nucleotides,[28] except in one unconfirmed instance with phosphate.[70]

[67] E. Volkin and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1519 (1951).

[68] J. R. Totter, E. Volkin, and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1521 (1951).

[69] P. Drochmans, D. H. Marrian, and G. B. Brown, *Arch. Biochem. and Biophys.* **39,** 310 (1952).

[70] P. Boulanger and J. Montreuil, *Biochim. et Biophys. Acta* **9,** 619 (1952).

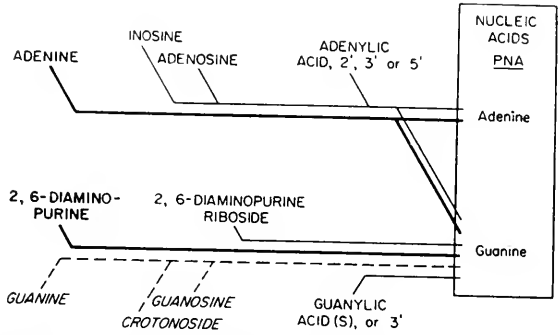

FIG. 1a. Purines and purine derivatives which can function as polynucleotide precursors in the rat.

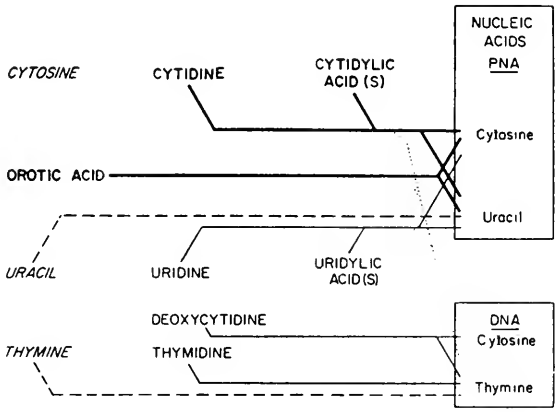

FIG. 1b. Pyrimidines and pyrimidine derivatives which can function as polynucleotide precursors in the rat.

The heavy lines indicate the most extensive incorporations, lighter lines moderate incorporation, and broken lines mere trace incorporation. The lines are intended to indicate only the overall conversions observed and no sequence of reactions is to be implied.

The dotted line indicates that appreciable transformations of those derivatives into deoxyribopolynucleotide components have been observed.

Fig. 1 depicts the array of precursors which have been shown to be capable of serving as precursors of the polynucleotides in the laboratory rat. In that outline the only distinctions which have been made are between those compounds which are very extensively incorporated, those which are definitely specific precursors but are incorporated to a more moderate extent, and those with which only a very small incorporation has been observed. In all cases except the one study with cytidine[65] and one with

guanylic acid,[70a] only the fate of the purine or pyrimidine moiety is specifically known.

4. Comparative Biochemistry of Purine Utilizations

The utilizations of various purines by a number of species have been studied. The examples, from among the mammals, bacteria, protozoa, and yeasts, show marked differences which cannot yet be correlated with any other characteristics of the organisms.

Among the purines studied, only two have been found to be extensively utilized by the rat: adenine[12,13] as a precursor of both adenine and guanine of the polynucleotides, and 2,6-diaminopurine[44,45] as a precursor of polynucleotide guanine only. However, in the C57 mouse guanine was incorporated[36,71] to a considerably greater extent, while adenine-1,3-N_2[15] was utilized[35] to a smaller extent than in the rat. In the mouse each purine is converted into the polynucleotide derivative of the other.[36,72]

In rabbit hyperplastic bone marrow slices, each purine is incorporated and each is converted into the polynucleotide derivative of the other to a small extent. In addition, free adenine, guanine, and hypoxanthine containing the label could be isolated after the administration of labeled adenine, which suggested[73] that interconversion of the free purines may occur, although it has been pointed out that the possibility of mediation of ribosides in the conversion is not excluded.[74]

The utilization of purines by a number of microorganisms has been studied, and different species present a whole spectrum of variations in their patterns of purine incorporations and interconversions. The range of variations in the extent to which the adenine and guanine are interconverted by several species is depicted graphically in Fig. 2.

In microorganisms with an obligate purine requirement, growth on a single purine has been taken as presumptive evidence that all of the polynucleotide purines arise from that single purine. However, organisms which do not require preformed purines for growth may readily utilize conveniently available purines in competition with the usual pathways of synthesis *de novo*; the use of isotopically labeled purines makes possible a direct demonstration of their incorporation, and is the only possible direct measure of the extent to which they are interconverted. In some instances the sparing effect of added purines on the synthesis *de novo* from labeled precursors (other than purines) has furnished[33,40,79,81] a reliable but less direct measure of the utilization

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[70a] P. M. Roll, H. Weinfeld, and G. B. Brown, *Biochim. et Biophys. Acta*, **13**, 141 (1954).

[71] G. B. Brown, *J. Cellular Comp. Physiol.* **38**, Suppl. 1, 121 (1951).

[72] H. G. Mandel and P. E. Carlo, *J. Biol. Chem.* **201**, 335 (1953).

[73] R. Abrams and J. M. Goldinger, *Arch. Biochem. and Biophys.* **30**, 261 (1951).

[74] H. M. Kalckar, *Fortschr. Chem. org. Naturstoffe* **9**, 363 (1952).

of the purine. In *L. casei* a "sparing" by purines of the synthesis *de novo*[85] is dependent[40] on the concentrations of both the purine and the folic acid in the medium. Thus the composition of the media may influence the incorporations (and the conversions) observed. The comparisons referred to here are mostly based upon the addition of equimolar quantities of different purines, or mixtures of purines, to a given medium for each organism.

At one extreme *Torulopsis utilis*[26,75] and *Aerobacter aerogenes*[76] readily incorporate either adenine or guanine. They extensively convert adenine into polynucleotide guanine, but they convert guanine to only a very small extent. Other species range, with a proportionately greater conversion of guanine into polynucleotide adenine, through two strains of *Paramecium aurelia*,[76,77] *Ochromonas malhamensis*,[78] and *E. coli*,[50,79] to *L. casei*,[40,80] which presents the maximum conversions of each purine into the polynucleotide derivative of the other. At the other extreme *Tetrahymena geleii*[83,86] and *L. leichmanii*[84] present the opposite picture. Each of these organisms readily incorporates either purine and converts guanine into polynucleotide adenine, but adenine was not found to be converted into polynucleotide guanine by either of these species.

The extent to which the interconversions are influenced by the presence of other purines also varies. In *T. utilis*[26,75] the amount of guanine derived from adenine is greatly reduced when guanine is present, while in *L. leichmanii*[84] the amount of adenine derived from guanine is reduced by the presence of adenine. In *L. casei*[40,80] when both purines are present the interconversion continues unabated and nearly half of each is still converted into the polynucleotide derivative of the other.

In the eight species where 2,6-diaminopurine has been tested,[40,44,45,75,77,78,81] it is transformed into polynucleotide guanine, sometimes more[45] and sometimes less[78] extensively than is guanine. It is also transformed into

[75] S. E. Kerr and F. Chernigoy, *J. Biol. Chem.* **200,** 887 (1953).
[76] L. D. Hamilton, G. B. Brown, and C. C. Stock, *J. Clin. Invest.* **31,** 636 (1952).
[77] C. C. Stock, M. Williamson, and W. Jacobson, *Ann. N. Y. Acad. Sci.* **56,** 1081 (1953).
[78] L. D. Hamilton, *Ann. N. Y. Acad. Sci.* **56,** 961 (1953).
[79] E. T. Bolton, P. H. Abelson, and E. Aldous, *J. Biol. Chem.* **198,** 179 (1952).
[80] M. E. Balis, G. B. Brown, G. B. Elion, G. H. Hitchings, and H. VanderWerff, *J. Biol. Chem.* **188,** 217 (1951).
[81] G. B. Elion, H. VanderWerff, G. H. Hitchings, M. E. Balis, D. H. Levin, and G. B. Brown, *J. Biol. Chem.* **200,** 7 (1953).
[82] G. B. Elion, S. Singer, G. H. Hitchings, M. E. Balis, and G. B. Brown, *J. Biol. Chem.* **202,** 647 (1953).
[83] M. Flavin and S. Graff, *J. Biol. Chem.* **191,** 55 (1951); **192,** 485 (1951).
[84] F. Weygand, A. Wacker, and H. Dellweg, *Z. Naturforsch.* **7b,** 156 (1952).
[85] H. M. Kalckar, *Harvey Lectures* **45,** 11 (1950–51).
[86] M. R. Heinrich, V. C. Dewey, and G. W. Kidder, *J. Am. Chem. Soc.* **75,** 1741 (1953).

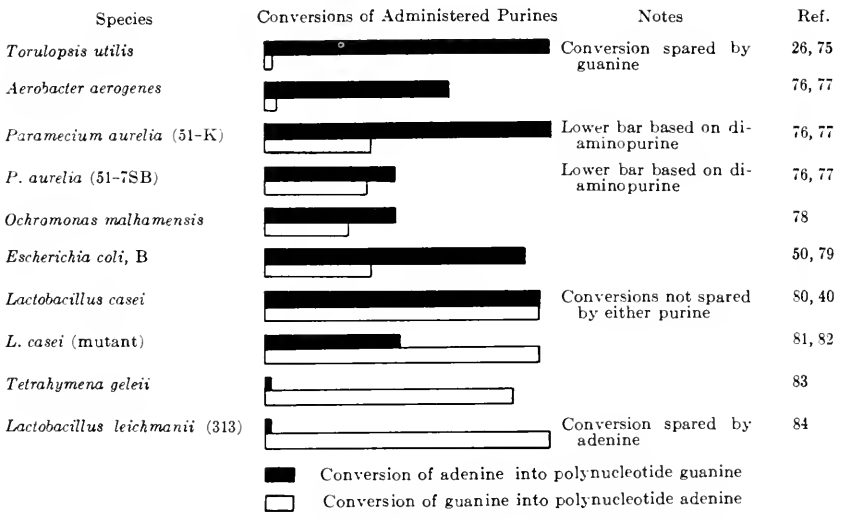

FIG. 2. Variations in the interconversions of administered purines by various species.

The lengths of the bars signify the amount of the polynucleotide purine which arises through conversion as a proportion of that arising through direct incorporation.

polynucleotide adenine to a degree quite similar to the conversions observed for guanine in the respective species.

When 2,6-diaminopurine is present with either or both of the other purines, it is transformed equally into each of the polynucleotide purines by *L. casei*,[40] and in mixtures of any two or three of the purines each is used to an extent approximately proportionate to its concentration.

There are instances where exogenous adenine or guanine is converted into the polynucleotide derivative of the other to a greater extent than it is incorporated per se. For example a haploid *Saccharomyces cerevisiae*[57] can convert adenine into PNA guanine to greater extent than it incorporates it as adenine. Guanine has not been tested in this particular yeast. In *L. casei* growing with adequate folic acid, the conversion of guanine into polynucleotide adenine seems slightly to exceed its direct incorporation.[40]

No obvious correlations seem to exist between the relative incorporations and conversions of a given purine. For instance, in *O. malhamensis*[78] guanine is incorporated twice as extensively as is adenine even though it is converted into PNA adenine less readily than adenine is converted into PNA guanine. Diaminopurine is utilized far less extensively but is transformed into adenine more extensively than is guanine.

It would appear that not only species but also strain differences in the patterns of purine utilization are possible. In a mutant of *L. casei* resistant to inhibition by diaminopurine, there was[81] a greatly decreased ability to utilize adenine or diaminopurine, accompanied by a reduced conversion of both. Two varieties of *P. aurelia*,

[87] R. Abrams, *Arch. Biochem. and Biophys.* **37**, 270 (1952).

the "killer" type (51-7K) and the kappa-free "sensitive" type (51-7SB), showed[77] a considerable difference in the extent to which adenine was converted into PNA guanine. Guanine was not tested in the paramecia, and in Fig. 2 the bars represent the relative transformation of 2,6-diaminopurine into adenine.

Only adenine has been tested in man *in vivo*.[88] It is utilized for the synthesis of both polynucleotide adenine and guanine of the leucocytes (in a chronic lymphatic leukemia). When human leukemic leucocytes were incubated in serum, adenine and guanine were utilized but were not interconverted, and diaminopurine was transformed into polynucleotide guanine only.

The meager evidence available on the utilization of nucleosides indicates that there are also species differences in the modes of metabolism of the ribosyl derivatives. The utilization of ribose derivatives with maintenance of the integrity of the ribosyl linkage is suggested by the specific growth response of some of the more fastidious organisms to pyrimidine or purine ribosides or ribotides,[89] and by the incorporation of pyrimidine ribosyl derivatives[20,28,39,64,65] by the rat. In *L. casei* several ribosides exhibit less facile interconversions than do the corresponding free purines (cf. Fig. 6), which suggests[32] that they are utilized as such and not via the free purines. The appearance of considerable amounts of the ribose of guanylic acid in the other nucleosides from the nucleic acids of *L. leichmanii*[65] indicates the occurrence of extensive transglycosidation (Chapter 24). In *E. coli* B the cytosine of cytidine is incorporated independently of its ribosyl moiety, with but small amounts of isotope appearing in any of the polynucleotide ribose.

III. Comparative Incorporations into Various Nucleic Acid Fractions

The ability to detect the metabolic replacement or renewal, of cell constituents is a unique virtue of isotopic tracers, but a word of caution is desirable regarding the dangers of overinterpretation of data thus accumulated. The incorporations observed are not only the result of both formation of new labeled molecules and the concurrent loss of some of these during the period of the experiment, but are also affected by the various equilibria in which the series of intermediates, through which the precursors must pass en route to the final products, are involved. Precursors of different degrees of complexity may enter a single assembly line at widely separated points, and the time courses of the incorporations of two precursors may be quite different by virtue of the fact that the one may pass into a slowly renewed intermediate, while the other may by-pass that intermediate and be incorporated only into rapidly renewed intermediates. The relative extents to which precursors, particularly when introduced in excess of physiological concentrations, are shunted into catabolic, or alternate anabolic, pathways can also influence the dilution factors. It is to be hoped

[88] L. D. Hamilton, *Nature* **172,** 457 (1953).
[89] W. S. McNutt, *Fortschr. Chem. org. Naturstoffe* **9,** 401 (1952).

that knowledge of the sequence of intermediates involved and detailed kinetic studies will eventually clarify some of the relationships.

In considering the available data on the relative incorporations of various precursors into nucleic acids, it must be kept in mind that they are collected under varying experimental conditions and that conclusions derived from comparisons of such data may not always be valid. It must also be kept in mind that the present results all represent averages for the nucleic acid "preparations" studied, and that any of the preparations may be susceptible to subfractionation. Individual components of the nucleic acids are observed to be renewed to different extents, and it is necessary to recognize which moiety of the molecule is to be considered. With multicomponent molecules such as the nucleic acids, the metabolic behavior of the molecules as a whole cannot yet be adequately deciphered, since the differences in incorporations observed may reflect either alternatives in the pathways leading to the immediate precursor or metabolic properties of the final molecules per se. It is also now known that the composition of the diet can influence at least liver PNA phosphorus renewal.[90] Such conclusions as now appear evident may require frequent reinterpretation as additional knowledge becomes available.

1. Relative Incorporations into Various Tissues

a. Incorporations into DNA's of Individual Organs

In their pioneer studies Hevesy and co-workers[91-93] found that the incorporation of P^{32}-labeled inorganic phosphate into DNA varied widely in different organs of the rat. It was found (Table II, columns 1–3) that the DNA of intestine and spleen were renewed much more rapidly than were those of several other organs. Even greater renewals were observed in the bone marrow and thymus, and the renewal in the lymphoid tissues was found to decrease with the age of the rats.[94] It was recognized that the greatest renewals were to be found in those organs where maximal new cell production occurs.

It was shown that different values for relative "renewals" would be obtained depending upon whether the results were expressed in terms of activities relative to a single reference value, or relative to the inorganic phosphate of the respective organs (compare Table II, columns 1 and 2,

[90] H. N. Munro, D. J. Naismith, and T. W. Wirkramanayake, *Biochem. J.* **54,** 198 (1953).

[91] L. Hahn and G. Hevesy, *Nature* **145,** 549 (1940).

[92] L. Ahlström, H. von Euler, and G. Hevesy, *Arkiv Kemi, Minerol. Geol.* **A19,** No. 9 (1945).

[93] G. Hevesy and J. Ottesen, *Acta Physiol. Scand.* **5,** 237 (1943).

[94] E. Andreasen and J. Ottesen, *Acta Physiol. Scand.* **10,** 258 (1945).

TABLE II

INCORPORATIONS OF CERTAIN PRECURSORS INTO THE NUCLEIC ACIDS OF VARIOUS ORGANS[a]

Precursor administered:	Incorporations into DNA																		Incorporations into PNA			
	Phosphate-P^{32}										Adenine-N^{15} and Formate-C^{14}				Formate-C^{14}						Formate-C^{14}		For-mate-C^{14}	
	1		2		3		4	5	6		7			8	9			10			11		12	
Species: Cond.: route times[b]	Rat i.p. 2h.		Rat i.p. 2h.		Rat i.p. 4d.		Rat i.p. 4h.	Mouse i.p. 2h.	Man sub. q. ly., 3d.		Rat i.p., simult. 1d.				Rat i.p. 3d., ld.						Rat i.p. ld.		Rat i.p. 3d., ld.	
Product studied:	DNA-P		DNA-P		DNA-P		DNA-P	DNA-P	DNA-P		A	A	G	T	DNA-1[c]			DNA-2[c]			Aden-ylic acid	Guan-ylic acid	A	G
															A	G	T	A	G	T				
Expressed as:	RA	Act.[d]	RA	Act.[d]	RA[e]		RA	RA	RA		RIC	c/m/μM.			c/m/μM.			c/m/μM.			c/m/μM.		c/m/μM.	
Ref.:	92		95		93		96	96	97		16				99						69		99	
Organs:																								
Small intestine	2.1[f]	4.8[f]	63	2.8	12.7[f]	15.4[f]	81.5	15.3			1.55	213	354	390	1850	1950	614	1920	1340	779	2775	2050	3310	2890
Large intestine and stomach											0.89	89	103	142										
Spleen	1.79	2.50	63	2.2	5.9	6.2	73.3	36.9	0.0159		0.37	39	44	41	595	505	255	574	590	293	900	1100	1800	1390
Nodes									0.0115															

Pancreas														383	326	113	354	337	107	350	150	714	625
Liver	0.20	0.14	4.4	0.105	1.01	1.66	2.25	1.01	0.0097	0.10	7	7	8	—	—	—	127	98	38	450	340	456	389
Testis					0.83	0.97					8	1	18	141	151	113	108	115	117	125	85	600	439
Kidney	0.15	0.16			0.60	0.67	1.2	0.54	0.0093		4	8	12	193	190	32	75	80	28	1620	775	2760	1230
Muscle					1.05	1.42			nil														
Cartilage									nil														
Brain					0.09	0.22			nil					0	0		0	0				116	90

[a] Throughout the tables certain of the experimental conditions are specified. Data are expressed in the terms used by the authors unless otherwise specified. A%E signifies atom per cent excess of heavy isotopes; c/m/μM. (or mg., etc.) signifies observed counts per minute per unit of materials. RIC and RSA signify relative isotope content and relative specific activities, and are equivalent to the per cent of *observed* renewal of the products.
They are defined as:

$$\text{RIC} = \frac{\text{atoms per cent excess in product}}{\text{atoms per cent excess in starting material}} \times 100$$

$$\text{RSA} = \frac{\text{activity per unit weight of sample}}{\text{activity per unit weight of starting material}} \times 100$$

In experiments with P^{32} relative activities (RA), proportionate to RSA's, are quoted here except as noted. The amount of total P administered is usually unknown and the results have been variously reported in terms of: the activity per mg. of the P of the product, the activity relative to that of the plasma inorganic phosphate, or per cent of the administered radioactivity per unit of P^{31} in the products studied.

In the table headings the following abbreviations have been used: m. = minute, h. = hour, d. = day, y. = year, i.p. = intraperitoneal, sub.q. = subcutaneous, A = adenine, G = guanine, T = thymine, C = cytosine, and U = uracil.

[b] Time interval following administration. Two figures represent the duration of the administration and the time interval following last injection.

[c] Values for two fractions[93] of the DNA from each organ.

[d] Activity relative to the activity of the inorganic P of the respective organs.

[e] Duplicate experiments.

[f] Mucosa.

TABLE III

INCORPORATIONS OF VARIOUS PRECURSORS INTO THE DNA OF INTESTINE AND LIVER (RAT)[a]

Prec. Admin.:	Ammonia-N^{15}	Glycine-N^{15}	Glycine-C^{14}	Glycine-C^{14} + Adenine-C^{13}				Phosphate-P^{32}	
Conditions:									
route	i.p.	sub.q.	i.p.	i.p.				sub.q.	
times[b]	4h., 32h.	6h., 6h.	24h., 12h.	24h., 12h.				24h.	48h.
Expressed as:	A%E	A%E	RSA	RSA				RA	RA
Product studied:	DNA-N	A G	A G	A	G	A	G	DNA-P	DNA-P
Ref.:	15	101	15	15				102	
Sm. intestine	0.142	0.27 0.48	2.69 2.81	1.55	1.93	8.7	3.2	7.34–5.92	6.52[c]
Liver	0.033	0.03 0.02	0.26 0.20	0.044	0.049	~0	~0	0.11	0.10

[a] Contractions used are explained in Table II, footnote *a*.

[b] Time interval following administration. Two figures represent the duration of the administration and the time interval following last injection.

[c] Mucosa only.

and Table IV, line 10 and footnote h). However, the only common basis yet available for comparisons of the phosphate data with other precursors is provided by the values relative to a single reference, since the results from each of the other compounds studied are compared to a single reference value (usually the activity of the administered compound).

Many subsequent studies with P^{32} or with adenine and formate have shown differences of the same order of magnitude in the relative incorporations into the DNA's of the majority of these several organs (Table II), with the renewals in intestine greater than those in spleen. However, in one case, that of strain A mice, the incorporation of phosphorus was repeatedly[96,100] found to be greater in spleen than in intestinal DNA (Table II, column 5), but in parallel experiments the incorporations of adenine, glycine, and formate were greater in the intestine than in the spleen.[100] Additional evidence on the relative incorporations into intestine and liver, including data for ammonia and glycine, are summarized in Table III. Although there may appear to be certain differences between the various

[95] E. Hammarsten and G. Hevesy, *Acta. Physiol. Scand.* **11,** 335 (1946).

[96] L. S. Kelly, A. H. Payne, M. R. White, and H. B. Jones, *Cancer Research* **11,** 694 (1951).

[97] E. E. Osgood, J. G. Li, H. Tivey, M. L. Duerst, and A. J. Seaman, *Science* **114,** 95 (1951).

[98] A. Bendich, *Exptl. Cell. Research.* **3,** Suppl. 2, 181 (1952).

[99] A. Bendich, P. J. Russel, Jr., and G. B. Brown, *J. Biol. Chem.* **203,** 305 (1953).

[100] A. H. Payne, L. S. Kelly, and H. B. Jones, *Cancer Research* **12,** 666 (1952).

[101] A. Bergstrand, N. A. Eliasson, E. Hammarsten, B. Norberg, P. Reichard, and H. von Ubisch, *Cold Spring Harbor Symposia Quant. Biol.* **13,** 22 (1948).

precursors, no generalizations seem to be justified from these data because of the varied experimental conditions and the technical difficulties involved in determining some of the smaller observed incorporations.

By comparing the specific activity of the phosphorus of the DNA with an average value throughout the experiment for the inorganic phosphorus of the organ, Hevesy and Ottesen[93] attempted to calculate minimum values for the daily renewal of the DNA-P for each organ. By such a calculation, it was concluded that about half of the newly formed DNA-P must represent "renewal" of the old molecules in a Jensen sarcoma.[10,103] More recently, a similar calculation of the daily renewal of DNA-P in rat liver and in intestine was based upon the total acid-soluble phosphorus of the organs, and was compared with mitotic counts as determined after colchicine arrest in metaphase.[102] The results led to the conclusion that the incorporation of the P^{32} represented twice as much synthesis of DNA as was required for new cell formation, and to the suggestion that each daughter cell receives a complete complement of new DNA. However such calculations may be subject to considerable error because of the difficulties in determining an average activity of the intercellular inorganic P over any appreciable period of time,[104-107] and the fact that it is now known that the inorganic P is not in facile equilibrium with the immediate nucleic acid precursors.[105,108]

In all of the data recorded in Tables II and III, there is a distinct parallel between the extent of cell division which is taking place in a tissue and the incorporation of any of these precursors into the DNA of that tissue.[10,16] This is particularly evident with intestine and spleen where the large incorporations parallel the extensive new cell formation, and in the low incorporations into muscle, cartilage, and brain where essentially no mitosis is taking place. In a man treated for a year with massive doses of P^{32}, no P^{32} was reported in the DNA of those tissues not undergoing mitosis (Table II, column 6). Thus, although there is evidence that some additional DNA synthesis or renewal may occur, the bulk of new DNA production does appear to take place in conjunction with new cell formation.

b. Incorporations into PNA's of Individual Organs

With all precursors the incorporations into PNA fractions exceed those into the DNA's. The relative incorporations of formate into the purine ribonucleotides or the PNA purines of several organs bear no apparent relationship to its incorporation into the DNA's of those same organs (Table

[102] C. E. Stevens, R. Daoust, and C. P. Leblond, *J. Biol. Chem.* **202,** 177 (1953).
[103] L. Ahlström, H. von Euler, G. Hevesy, and K. Zerahn, *Arkiv Kemi, Mineral. Geol.* **A23,** No. 10 (1946).
[104] C. Heidelberger, *Advances in Cancer Research* **1,** 273 (1953).
[105] C. P. Barnum and R. A. Huseby, *Arch. Biochem.* **29,** 7 (1950).
[106] D. B. Zilversmit, E. Enteman, and M. C. Fishler, *J. Gen. Physiol.* **26,** 325 (1953).
[107] J. Sacks, *Cold Spring Harbor Symp. Quant. Biol.* **13,** 180 (1948).
[108] R. M. S. Smellie, W. M. McIndoe, R. Logan, J. N. Davidson, and I. M. Dawson, *Biochem. J.* **54,** 280 (1953).

TABLE IV

INCORPORATIONS OF VARIOUS PRECURSORS INTO THE PNA OF INTESTINE AND LIVER (RAT)[a]

Precursor	Conditions: route, times[b]	Data expressed as:	Liver		Intestine		Liver/intestine ratios		Ref.
			A	G	A	G	A	G	
1. Formate-C^{14}	i.p., 1d.	c/m/μM.	450	340	2775	2050	0.16	0.16	69
2. Formate-C^{14}	i.p., 3d., 1d.	c/m/μM.	456	389	3310	2890	0.14	0.14	99
3. Ammonia-N^{15}	i.p., 4h., 32h.	A%E	0.045	0.090	0.179	0.338	0.25	0.27	15
4. Glycine-N^{15}	sub.q., 6h., 6h.	A%E	0.08	0.07	0.46 (0.51)[c]	0.51 (0.97)[c]	0.17	0.14	101 109
5. Glycine-N^{15}	i.p., 4h., 32h.	A%E	0.015	0.035	0.132	0.220	0.11	0.16	15
6. Glycine-1-C^{14}	i.p., 24h., 12h.	RSA	1.40	1.35	3.71	3.77	0.38	0.36	15
7. Glycine-1-C^{14} and Adenine-2-C^{13}	i.p., (si-mult.[d]) 24h., 12h.	RSA RIC	0.26 12.6	0.36 4.1	2.29 15.0	2.60 5.5	0.11 0.84	0.14 0.75	15
8. Formate-C^{14} and Adenine-1,3-N_2^{15}	i.p., (si-mult.[e]) 1d.	c/m/μM. RIC	63 4.3	43 0.7	254 2.6	277 0.60	0.25 1.65	0.16 1.2	16
9. Adenine-1,3-N_2^{15}	oral[f], 1d., 1d.	RIC[g]	8.7	3.7	4.5	1.4	1.9	2.6	17
10. Phosphate-P^{32}	i.p., 2h.	RA[h]	PNA-P 164		PNA-P 112		1.46		95

[a] Contractions used are explained in Table II, footnote *a*.

[b] Time interval following administration. Two figures represent the duration of administration and the time interval following last administration.

[c] N^{15} in hypoxanthine and xanthine derived by deamination.

[d] 1.9 mM. adenine per kg. per day.

[e] 0.2 mM. adenine per kg. per day.

[f] 0.4 mM. adenine per kg. per day.

[g] Of mixed nucleic acids.

[h] Relative to the specific activity of inorganic P of total rat; the values presented as relative to the specific activity of inorganic P of the respective organs have been most frequently quoted, these are 3.45 and 6.1, respectively.

II, columns 11 and 12). In view of the considerable metabolic (Chapter 26) and compositional (Chapters 11 and 18) differences between nuclear PNA and the several cytoplasmic PNA's it must be recognized that most of the data quoted in Tables II, IV, and V represents averages of the several

[109] P. Reichard, *J. Biol. Chem.* **179,** 773 (1949).

PNA's, although in some instances the nuclear PNA has been studied separately.

With formate the appreciable renewal of the PNA of brain indicates that the lack of incorporation into brain DNA in the same experiment (Table II, columns 9 and 10) does not need to be attributed to an impermeable barrier. The large renewal of the kidney PNA, coupled with the low renewal of its DNA, gives it the highest ratio between the renewals of PNA and DNA of any of these organs except brain. Small variations between the renewals observed in liver, pancreas, and testis, and the reversal of the ratio of incorporation into adenine and guanine in spleen in the two experiments remain unexplained.

For liver and intestinal PNA's the relative incorporations of several precursors are available (Table IV). There is a considerably greater incorporation of those precursors which are involved in the synthesis *de novo* of the carbon–nitrogen skeleton of the purines into the intestinal PNA than into the liver PNA, and this is in striking contrast to the approximately equal or greater incorporation of administered adenine into the PNA of the liver as compared to intestine. Thus with formate, ammonia, or glycine the ratios for the incorporation into the PNA of liver and of the intestine, either into the adenines or the guanines, range from 0.38 to 0.11. On the other hand, the corresponding liver-to-intestine ratios for the incorporation of adenine are 1.65 to 0.75. Two double-labeling experiments, one involving simultaneous administration of formate-C^{14} and adenine-1,3-N_2^{15} (line 8),[16] and the other glycine-1-C^{14} and adenine-2-C^{13} (line 7)[15] have been performed and each unequivocally confirms the differences in the behaviors of the respective purine precursors relative to that of the preformed purine. The absolute incorporation of adenine was slightly greater in the intestinal PNA in one investigation (line 7), but the fact that the quantity of adenine used there was 9.5 times that used in the other (line 8) may have been a factor. Verification of the facile incorporation of adenine into liver PNA also comes from comparison of lines 6 and 7, where the large dose of adenine administered brought about in the liver a far more pronounced sparing, or inhibition, of glycine utilization that it did in the intestine. With orotic acid, 16 % of the dose was found in rat liver PNA and but 1.5 % in the remaining viscera at 20 hours,[60] and it appears that this pyrimidine precursor is also readily incorporated into liver PNA. The behavior of phosphate (line 10) is, curiously enough, more analogous to that of adenine than to that of the smaller precursors.

The marked contrasts between the incorporations of preformed adenine and those of glycine and formate into liver and intestinal PNA's forcibly emphasize the fact that experiments with a single "tracer" cannot be relied upon to measure the intrinsic metabolic activity of even the purine moieties, much less of the whole nucleic acid molecule.

2. Relative Incorporations into PNA and DNA of Liver

a. Phosphate

The initial studies[10] with phosphate-P^{32} involved only its incorporation into DNA, but in 1944 Brues *et al.*[110] investigated the incorporation into both the PNA and the DNA of rat liver. Three of eight days after injection they found a 5- to 6-fold greater incorporation of the P^{32} into the PNA in normal rat liver (Table V, p. 368, lines 1 and 2). However, in liver which was regenerating after partial hepatectomy, they found that after 3 days the ratio of the renewals of the PNA and the DNA was only 1.3, in accord with the evidence that there is an increased synthesis of DNA in the growing tissue, and after 13 days the activity of the PNA had dropped below that of the DNA to give a ratio of 0.6, which was the first evidence to suggest that the phosphorus of the DNA synthesized during growth was retained longer than was that of the PNA (Table V, lines 10 and 11). Hammersten and Hevesy[95] found, 2 hours after P^{32} administration, a liver PNA:DNA ratio of about 33 (27 to 40) (Table V, line 4) (but only 3 for spleen and 2 for intestine, lines 16 and 17). At 2 hours Davidson[111] found a ratio of 7 (line 3) and also the decrease in the ratio in regenerating liver (line 12). Other values for regenerating liver (lines 13–15), and for fetal liver (lines 34–37) also show the lower ratios.

With improvements in the techniques (cf. Chapters 10 and 11) of separating the PNA from the DNA and of freeing the DNA from other highly active contaminants, the ratios obtained tend to be higher. On the other hand, improvements in the separation of the PNA fractions from more highly active phosphorus-containing compounds tend to result in lower ratios. Subsequent experiments on the incorporation of phosphate into liver nucleic acids in several species, covering periods of a few hours to a day or more (Table V, lines 5–9, 18, 19, 21–33, 48, and 49), have yielded various ratios which sometimes exceed the 33 of Hammersten and Hevesy, and even higher ratios for the relative incorporations into nuclear PNA (nPNA) and the DNA (most notably, lines 8, 9, and 25). Certain low PNA:DNA ratios obtained with chromatographically separated nucleotides[67] (Table V, lines 13 and 21) were based upon a high-molecular-weight PNA which represented only a portion of the "soluble" PNA of the cytoplasm.[125]

[110] A. M. Brues, M. M. Tracy, and W. E. Cohn, *J. Biol. Chem.* **155,** 619 (1944).

[111] J. N. Davidson, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 50 (1947).

[112] J. N. Davidson, S. C. Frazer, and W. C. Hutchison, *Biochem. J.* **49,** 311 (1951).

[113] J. N. Davidson, R. Logan, E. R. M. Kay, and R. M. S. Smellie, unpublished results.

[114] R. M. S. Smellie, W. M. McIndoe, and J. N. Davidson, *Biochim. et Biophys. Acta* **11,** 559 (1953).

[115] A. H. Payne, L. S. Kelly, G. Beach, and H. B. Jones, *Cancer Research* **12,** 426 (1952).

The data for other organs (many of which were contributed by R. M. S. Smellie, and which are discussed in more detail in Chapter 26) have, with the possible exception of kidney (lines 46 and 47), shown generally lower ratios than those for liver. The recognition of the intracellular heterogeneity of PNA decreases the significance of comparison of the total PNA with the DNA. However, with precursors other than phosphorus, few separations of PNA fractions have yet been made, and the bulk of the data now available for consideration deals with total PNA, or with but a few of the PNA fractions.

b. Precursors of the Carbon-Nitrogen Skeleton

Ammonia (Table V, lines 88–90) is also incorporated into PNA to a greater extent than into the DNA but the ratios observed are smaller. Bergstrand *et al.*[101] and Eliasson *et al.*[126] showed that the nitrogen of glycine-N^{15} was incorporated into the DNA purines of rat livers to a smaller extent than into the PNA purines, and that the nPNA fraction is renewed more rapidly than is the cytoplasmic PNA (cPNA). Numerous subsequent experiments with glycine, variously labeled in the nitrogen or in either of its carbons, have always shown this significant incorporation into the DNA of liver and have yielded PNA:DNA ratios ranging from about 3 to 8 (Table V, lines 65–67, 69–71, 74–76, 85, 103, 107, and 111), although recently some much higher ratios have been obtained with glycine (lines 77 and 78). It was also shown that, as with phosphate, the purines of the DNA fraction from regenerating liver show a greater incorporation of the glycine nitrogen and a resultant lowering of the PNA:DNA ratio (Table V, lines 68, 79, 80, and 105). Investigations of the relative incorporations of formate into liver nucleic acids have shown (Table V, lines 51–63, 109) ratios of the order found with glycine, including an increased incorporation into DNA in regenerating liver (lines 58 and 59).

In contrast to the smaller precursors, the incorporation of administered adenine into the purines of the PNA of rat liver has been found to exceed

[116] P. Reichard, *Acta Chem. Scand.* **3,** 422 (1949).
[117] W. M. McIndoe and J. N. Davidson, *Brit. J. Cancer* **6,** 200 (1952).
[118] G. A. LePage and C. Heidelberger, *J. Biol. Chem.* **188,** 593 (1951).
[119] S. S. Furst and G. B. Brown, *J. Biol. Chem.* **191,** 239 (1951).
[120] A. C. Griffin, W. E. Davis, Jr., and M. O. Tifft, *Cancer Research* **12,** 707 (1952).
[121] D. H. Marrian, *Biochim. et Biophys. Acta* **13,** 282 (1954).
[122] J. R. Fresco and A. Marshak, *J. Biol. Chem.* **205,** 585 (1953).
[123] E. P. Tyner, C. Heidelberger, and G. A. LePage, *Cancer Research* **13,** 186 (1953).
[124] E. P. Anderson and S. E. G. Åqvist, *J. Biol. Chem.* **202,** 513 (1953).
[125] E. Volkin, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 338. Johns Hopkins Press, Baltimore, 1952.
[126] N. A. Eliasson, E. Hammarsten, P. Reichard, S. E. G. Åqvist, B. Thorell, and G. Ehrensvärd, *Acta Chem. Scand.* **5,** 431 (1951).

that into the purines of the DNA by a much greater margin,[17] with values for the PNA:DNA ratio ranging from 35 to 73 (Table V, lines 91–101, 104, 108, and 110). The pyrimidine precursor, orotic acid, also results[60] in PNA:DNA ratios of the magnitude found with adenine (lines 102 and 113).

Throughout the period of regeneration of the liver of partially hepatectomized rats, a greatly increased incorporation of adenine into the DNA fraction resulted in PNA:DNA ratios of only 1.4 to 2.6 (Table V, lines 92 and 106) and demonstrated that it was not an inability to utilize adenine for DNA synthesis which was responsible for the high ratios in normal liver. When similarly treated rats were allowed to survive for a period of time, the isotope which had been incorporated into the DNA purines during the period of rapid growth of the liver was extensively retained, while that in the PNA was largely lost, which resulted in PNA:DNA ratios of 0.3 after 21 days[16] and 0.01 at 96 days[119] (lines 93 and 96). Later[98,99] the retention of the isotope of formate-C^{14} which was incorporated into the various bases of the DNA during the period of regeneration of liver was found to be appreciably greater than was the retention of that incorporated into normal liver (Table V, line 59, and Table VI).

It should be noted that in the intestine all of the precursors, glycine (Table V, lines 72, 73, 83, 84, and 118), formate (Table V, line 120, and Table II), adenine (Table V, lines 117 and 119), and phosphate (lines 17, 42, and 43), result in PNA:DNA ratios of approximately 1.5 to 2, so that the incorporations into this rapidly growing tissue are analogous to those in regenerating liver.

The marked contrasts between the incorporations of the various precursors into liver PNA and DNA are amply confirmed by the several experiments cited in Table V, including several instances where simultaneous administration of the two precursors labeled with different isotopes furnished direct comparisons under identical experimental conditions (lines 103–120). This was done with glycine and adenine in two laboratories;[15,115] with formate and adenine;[16] with glycine and phosphate;[123] and with orotic acid and phosphate.[124] The incorporations of four precursors into gross nucleic acid fractions were also compared under similar conditions[100] (lines 22, 23, 60, 61, 69, and 97).

There is thus a generally good agreement that lower ratios are obtained with glycine or formate and higher ratios with adenine, orotic acid, and P^{32}.

In view of the findings[124] that the relative incorporations of simultaneously administered orotic acid and phosphate into liver nucleic acid fractions are not fully parallel, it may be that they and adenine are incorporated at similar, but not identical, stages of the biosynthesis pathway. It was noted[123] that, when administered simultaneously, relatively more phosphate than glycine carbon appeared in the purine nucleotides of the PNA at the shorter time intervals. The phosphorus which

finally reaches the polynucleotides may not be that which is introduced in the original synthesis *de novo* of a purine derivative (Chapter 23) but may be one which, like adenine or orotic acid, is introduced at some later stage in the assembly line.

The existence[15,69] of facile incorporation of administered adenine for the synthesis of liver PNA's (Table IV) may furnish a partial explanation of the enhanced liver PNA : DNA ratios obtained with it, and a similar factor may play a role with orotic acid.[60] The observations that the purine ribosides or ribotides are not incorporated into DNA of the total viscera to a great extent, although corresponding pyrimidine ribosyl derivatives do lead to a considerable renewal of the DNA polynucleotide pyrimidines, cannot yet be interpreted since the incorporations of those ribosyl derivatives into individual organs have not yet been studied.

The proportionately greater incorporation of all precursors into the DNA of regenerating liver does augment the evidence in Table II that a more extensive DNA synthesis is associated with mitosis. Furst *et al.*[17,119] emphasized that the incorporation of adenine was extremely small in the absence of extensive mitosis, and, in particular, that the long-term retention of the isotope once incorporated demonstrated a pronounced biochemical stability of the bulk of the DNA, and that such a property may be pertinent to the candidacy of DNA for a role as a part of the genetic material. The proteins of the nucleus have been demonstrated by Hammarsten and co-workers[101,126,127] to be extensively renewed by glycine nitrogen, and Daly *et al.*[128] have carried this one step further and showed that the residual protein is renewed more extensively than is the histone, but no protein has yet displayed a nondynamic character comparable to that of the bulk of the DNA.

The deductions regarding the quantity of DNA renewed in a growing tumor,[10,103] or in normal liver and intestine,[102] show that the synthesis of DNA is in excess of that required for new cell production and suggest some renewal of DNA. The relative incorporations observed for glycine, formate, and serine in nondividing tissues have frequently been considered[39,68,73,109,118,129-131] to be incompatible with the evidence suggesting a comparatively limited turnover of DNA. Initially, these differences in the relative incorporations of glycine or formate and adenine, and even the smaller differences between adenine and phosphate, could be taken[132] as suggestive that individual moieties of the polynucleotides, or at least of their immediate precursors, might be renewed independently. Specific investigations, involving degradations to locate the positions of the isotope, of the possible indepen-

[127] E. Hammarsten, *in* "Isotopes in Biochemistry" (Wolstenholme, ed.), p. 203. Blakiston, Philadelphia, 1951.
[128] M. M. Daly, V. G. Allfrey, and A. E. Mirsky, *J. Gen. Physiol.* **36,** 173 (1952).
[129] D. Elwyn and D. B. Sprinson, *J. Am. Chem. Soc.* **72,** 3317 (1950).
[130] E. P. Tyner, C. Heidelberger, and G. A. LePage, *Cancer Research* **12,** 158 (1952).
[131] J. Brachet, *Actualités biochim.* No. 16 (1952).
[132] G. B. Brown, *Cold Spring Harbor Symposia Quant. Biol.* **13,** 43 (1948).

TABLE V

RATIOS OF INCORPORATIONS OF VARIOUS PRECURSORS INTO THE PNA AND DNA OF DIFFERENT TISSUES

	Precursor	Tissue	Time of experiment[a]	Product analyzed	Origin of PNA[b, c]	PNA:DNA[d]	Ref.
1	Phosphate	Rat liver	3 days	Nucleic acid phosphorus (NA-P)		5.2	110
2	Phosphate	Rat liver	8 days	NA-P		5.9	110
3	Phosphate	Rat liver	2 hr.	NA-P	cPNA	7.2	111
4	Phosphate	Rat liver	2 hr.	NA-P		33	95
5	Phosphate	Rat liver	2 hr.	Nucleotides[e]	cPNA	1.5–2.7	108
6				Nucleotides[e]	nPNA	34–42	108
7	Phosphate	Rat liver	4 hr.	Nucleotides[e]	cPNA	6–8	108
8				Nucleotides[e]	nPNA	100	108
9	Phosphate	Rat liver	2 hr.	Nucleotides[e]	nPNA	64–100	117
10	Phosphate	Regenerating rat liver	3 days	NA-P		1.3	110
11	Phosphate	Regenerating rat liver	13 days	NA-P		0.6	110
12	Phosphate	Regenerating rat liver	2 hr.	NA-P	cPNA	1.6–2.7	111
13	Phosphate	Regenerating rat liver	⅓ hr.	Nucleotides		2–4	67
14	Phosphate	Regenerating rat liver	4 hr.	Nucleotides[e]	cPNA	1.6–2	108
15				Nucleotides[e]	nPNA	14–16	108
16	Phosphate	Rat spleen	2 hr.	NA-P		3	95
17	Phosphate	Rat intestine	2 hr.	NA-P		2	95
18	Phosphate	Weanling rat liver	4 hr.	Nucleotides[e]	cPNA	2.4	108
19				Nucleotides[e]	nPNA	25	108

20	Phosphate	Rat hepatoma	3 days	NA-P		2.7	110
21	Phosphate	Mouse liver	⅓ hr.	Nucleotides		6–10	67
22	Phosphate	Mouse liver	4 hr.	NA-P	cPNA	14.2	100
23				NA-P	nPNA	56.7	100
24	Phosphate	Mouse liver	24 hr.	NA-P	cPNA	27–37	105
25		Mouse liver			nPNA	100	105
26	Phosphate	Mouse liver	24 hr.	NA-P	cPNA	16.3	100
27	Phosphate	Rabbit liver	2 hr.	NA-P	cPNA	7	112
28	Phosphate	Rabbit liver	4 hr.	Nucleotides[e]	cPNA	1.7	108
29				Nucleotides	nPNA	19	108
30	Phosphate	Rabbit liver	18 hr.	Nucleotides[e]	cPNA	8.4–8.9	113
31				Nucleotides[e]	nPNA	12.8–13.8	113
32	Phosphate	Maternal rabbit liver	4 hr.	Nucleotides[e]	cPNA	0.8	108
33	Phosphate			Nucleotides[e]	nPNA	14.6	108
34	Phosphate	Fetal rabbit liver	4 hr.	Nucleotides[e]	cPNA	0.3	108
35	Phosphate			Nucleotides[e]	nPNA	2.5	108
36	Phosphate	Fetal rat liver	2 hr.	NA-P	cPNA	0.2	111
37	Phosphate	Fetal rat liver	4 hr.	NA-P	cPNA	1.2	111
38	Phosphate	Rabbit thymus	18 hr.	Nucleotides[e]	cPNA	1.2–1.3	113
39				Nucleotides[e]	nPNA	1.4–1.6	113
40	Phosphate	Rabbit bone marrow	18 hr.	Nucleotides[e]	cPNA	0.8–0.9	113
41	Phosphate			Nucleotides[e]	nPNA	0.7–0.8	113
42	Phosphate	Rabbit intestinal mucosa	18 hr.	Nucleotides[e]	cPNA	1.5–1.9	113
43	Phosphate			Nucleotides[e]	nPNA	1.4–1.8	113
44	Phosphate	Rabbit appendix	18 hr.	Nucleotides[e]	cPNA	0.8–1.0	113
45	Phosphate			Nucleotides[e]	nPNA	0.8–0.9	113
46	Phosphate	Rabbit kidney	18 hr.	Nucleotides[e]	cPNA	25–26	113
47	Phosphate			Nucleotides[e]	nPNA	32–40	113
48	Phosphate	Fowl liver[f]	2 hr.	Nucleotides[e]	nPNA	27–34	117
40	Phosphate	Fowl liver[f]	2 hr.	Nucleotides[e]	cPNA	2.1–3.1	117
50	Formate-C^{14}	Rat viscera	16 hr.	Adenylic acid		2.1	68
51	Formate-C^{14}	Rat liver	16 hr.	Nucleotides		1.4–2.3	68

TABLE V.—(*Continued*)

	Precursor	Tissue	Time of experiment[a]	Product analyzed	Origin of PNA[b, c]	PNA:DNA[d]	Ref.
52	Formate-C^{14}	Rat liver	24 hr.	Purines		3.6; 4[g]	99
53	Formate-C^{14}	Rat liver	24 days	Purines		3.0; 1.6[g]	99
54	Formate-C^{14}	Rat liver	4 hr.	NA-fractions	cPNA	4.9	114
55	Formate-C^{14}			NA-fractions	nPNA	7.9	114
56	Formate-C^{14}	Rat liver	16 hr.	NA-fractions	cPNA	2.2	114
57	Formate-C^{14}			NA-fractions	nPNA	5.5	114
58	Formate-C^{14}	Regenerating rat liver	24 hr.	Purines		1.0–1.4[g]	99
59	Formate-C^{14}	Regenerating rat liver	24 days	Purines		0.1–0.2[g]	99
60	Formate-C^{14}	Mouse liver	4 hr.	NA-fractions	cPNA	3.0	100
61	Formate-C^{14}			NA-fractions	nPNA	10	100
62	Formate-C^{14}	Mouse liver	24 hr.	NA-fractions	cPNA	16.3	100
63	Formate-C^{14}	Mouse liver[f]	4 hr.	NA-fractions	cPNA	1.1	115
64	Formate-C^{14}	Chicken viscera	16 hr.	Nucleotides		4.8	68
65	Glycine-2-C^{14}	Rat liver[f]	48 hr.	Adenine		1.0–4.5	118
66	Glycine-2-C^{14}	Rat liver	48 hr.	Guanine		3.7	53
67	Glycine-2-C^{14}	Rat liver[f]	18 hr.	Guanine		2.5	53
68	Glycine-2-C^{14}	Regenerating rat liver	48 hr.	NA-fractions		0.9–1.5	118
69	Glycine-2-C^{14}	Mouse liver	4 hr.	NA-fractions	cPNA	3.2	100
70	Glycine-2-C^{14}	Mouse liver[f]	4 hr.	NA-fractions	cPNA	2.1	115
71	Glycine-1-C^{14}	Rat liver	16 hr., 20 hr.	Purines		5.4; 6.7	15
72	Glycine-1-C^{14}	Rat intestine	16 hr., 20 hr.	Purines		1.4–1.3	15
73	Glycine-1-C^{13}	Rat intestine	16 hr., 20 hr.	Purines		1.4	15
74	Glycine-N^{15}	Rat liver	6 hr., 6 hr.	Purines	cPNA	3–4	101
75	Glycine-N^{15}	Rat liver	6 hr., 6 hr.	Purines	nPNA	5–6	101
76	Glycine-N^{15}	Rat liver	5 days	Adenine		2.6	119
77	Glycine-N^{15}	Rat liver	4 hr., 20 hr.	NA-fractions	cPNA	20	114
78	Glycine-N^{15}	Rat liver		NA-fractions	nPNA	70	114

79	Glycine-N^{15}	Regenerating rat liver	6 hr., 6 hr.	Purines	cPNA	1.4	101
80	Glycine-N^{15}	Regenerating rat liver	6 hr., 6 hr.	Purines	nPNA	2	101
81	Glycine-N^{15}	Rat spleen	6 hr., 6 hr.	Adenine		1.7	116
82	Glycine-N^{15}	Rat kidney	6 hr., 6 hr.	Adenine		1.2	116
83	Glycine-N^{15}	Rat intestine	6 hr., 6 hr.	Purines		1.7; 1.1	109
84	Glycine-N^{15}	Rat intestine	3 hr., 3 hr.	Guanine		2.0	15
85	Glycine-N^{15}	Mouse liver	9 hr.	NA-fractions	cPNA	8.6	128
86	Glycine-N^{15}	Mouse kidney	9 hr.	NA-fractions	cPNA	17.1	128
87	Glycine-N^{15}	Mouse pancreas	9 hr.	NA-fractions	cPNA	2.7	128
88	Ammonia-N^{15}	Rat intestine	3 hr., 3 hr.	Purines		2.0; 1.9	15
89	Ammonia-N^{15}	Rat liver	3 hr., 3 hr.	NA-fractions		8.1	15
90	Ammonia-N^{15}	Pigeon liver	3 days	NA-fractions		16	111
91	Adenine-1,3-N^{15}	Rat liver	5 days, 0 hr.	Adenine		73	17
92	Adenine-1,3-N^{15}	Regenerating rat liver	5 days, 0 hr.	Purines		1.4; 2.6	17
93	Adenine-1,3-N^{15}	Regenerating rat liver	5 days, 21 days	Purines (mixed)		0.3	17
94	Adenine-1,3-N^{15}	Rat viscera	10 days.	Purines		29–24	14
95	Adenine-8-C^{14}	Rat liver	24 hr.	NA-fractions	nPNA	>100	120
96	Adenine-8-C^{14}	Regenerating rat liver	3 days, 96 days	Adenine		<0.01	119
97	Adenine-4,6-C^{14}	Mouse liver	4 hr.	NA-fractions	cPNA	15	100
98	Adenine-4,6-C^{14}	Mouse liver	24 hr.	NA-fractions	cPNA	24.8	100
99	Adenine-8-C^{14}	Rat liver	3 days	Purines		35; 37	121
100	Adenine-C^{14}	Mouse liver	3 hr.	Purines	nPNA	20; >35	122
101	Adenine-C^{14}	Mouse liver	3 hr.	Purines	cPNA	5; —	122
102	Orotic acid-C^{14}	Rat liver[f]	20 hr.	Pyrimidines		60	60
		Two labeled precursors administered simultaneously					
103	Glycine-N^{15}	Rat liver	5 days, 0 hr.	Purines		2.6; 3.2	119
104	Adenine-8-C^{14}	Rat liver		Purines		60; 41	119
105	Glycine-N^{15}	Regenerating rat liver	5 days, 0 hr.	Purines		0.9; 1.1	119
106	Adenine-8-C^{14}	Regenerating rat liver		Purines		2.6; 2.6	119

TABLE V.—(*Continued*)

	Precursor	Tissue	Time of experiment[a]	Product analyzed	Origin of PNA[b, c]	PNA:DNA[d]	Ref.
107	Glycine-1-C^{14}	Rat liver	16 hr., 20 hr.	Purines		5.7; 7.3	15
108	Adenine-2-C^{13}	Rat liver		Purines		38	15
109	Formate-C^{14}	Rat liver	24 hr.	Purines		9	16
110	Adenine-1,3-N^{15}	Rat liver		Purines		50	16
111	Glycine-2-C^{14}	Rat liver	12 hr.	Nucleotides		2–3[h]	123
112	Phosphate	Rat liver		Nucleotides		6–10[h]	123
113	Orotic acid-N^{15}	Rat liver	2 hr.	Pyrimidines	cPNA[i]	18–22	124
114	Phosphate	Rat liver		Nucleotides	cPNA[i]	6–9	124
115	Orotic acid-N^{15}	Regenerating rat liver	2 hr.	Pyrimidines	cPNA[i]	18–46	124
116	Phosphate	Regenerating rat liver		Nucleotides	cPNA[i]	14–18	124
117	Adenine-2-C^{13}	Rat intestine	16 hr. 20 hr.	Purines		1.7	15
118	Glycine-1-C^{14}	Rat intestine		Purines		1.3–1.5	15
119	Adenine-1,3-N^{15}	Rat intestine	24 hr.	Purines		1.6–1.5	156
120	Formate-C^{14}	Rat intestine		Purines		1.4–0.7	156

[a] Time interval following administration. Two figures represent the duration of administration and the time interval following the last injection.

[b] cPNA is equivalent to cytoplasmic PNA:DNA.

[c] nPNA is equivalent to nuclear PNA:DNA.

[d] Where two values are separated by a semicolon, they are the ratios for the respective adenines and guanines; otherwise they represent a range. In some instances, the ratios have been calculated from the authors' values.

[e] Range for individual ribonucleotides compared to total DNA-P.

[f] Tumor-bearing animals.

[g] Range of several values from two fractions of DNA.

[h] Approximate values abstracted from log plots.

[i] For nPNA:DNA the ratios are over 300 and 150, respectively.

TABLE VI

METABOLIC INHOMOGENEITY OF DNA[a, b]

Apparent retentions over a 23-day period of C^{14} (from formate) in the nucleic acids of individual organs.[99]

	PNA		DNA-1[c]			DNA-2[c]		
	A	G	A	G	T	A	G	T
1. Small Intestine	1.9	2.7	2.4	2.1	4.1	2.7	3.6	4.2
2. Spleen	5	8	11	15	20	6	15	22
3. Pancreas	24	21	42	40	52	35	42	38
4. Kidney	13	41	39	65	66	92	116	93
5. Testis	25	31	82	68	85	105	89	62
6. Liver, normal	10	13		—[d]		15	33	53
7. Liver, regenerating	7	13	55	56	92	57	82	85
8. Brain	46	51		—[e]			—[e]	

[a] A = adenine, G = guanine, T = thymine.

[b] The isotope content of each product on the 24th day is expressed as per cent of the corresponding value on the 1st day. The absolute isotope contents of each product on the 1st day are to be found in Table II, columns 9, 10, and 12.

[c] Two fractions of the DNA which differ in their ease of sedimentability in 0.87% NaCl.[98]

[d] None of this fraction was obtained from normal liver.

[e] No initial incorporation was observed.

dent renewal of carbons 2 or 8 of the ring of the polynucleotide purines by formate,[51,52,133] or by the α-carbon of glycine[52] did not lend support to this argument. The incorporation of phosphate has frequently shown[67,108,123,134] small differences in the renewal of the phosphorus of individual nucleotides, but these differences are from nucleic acid fractions which may well represent mixtures of molecular entities of varying compositions and cannot be considered as proof that individual moieties of a given macromolecule are renewed independently.

Numerous suggestions have been put forward[15,118,119,123,135] to explain the differences in the relative utilizations of the two types of precursors. The consideration of an impermeability of the nuclear envelope to adenine except during mitosis[15] must, in view of the ready incorporation of adenine into nuclear PNA (Table V, lines 95 and 100), be at least revised to specify an adenine-containing DNA precursor. The suggestions which now seem most readily susceptible to experimental approach are the possibilities of a heterogeneity of DNA, or that of two mechanisms of synthesis.

The extent of metabolic inhomogeneity of DNA thus far demonstrated (Table VI)

[133] W. H. Marsh, Thesis, Western Reserve University, 1951.

[134] T. Hultin, D. B. Slautterback, and G. Wessel, *Exptl. Cell Research* **2**, 696 (1951).

[135] G. B. Brown, *in* "Isotopes in Biochemistry" (Wolstenholme, ed.), p. 164. Blakiston, Philadelphia, 1951.

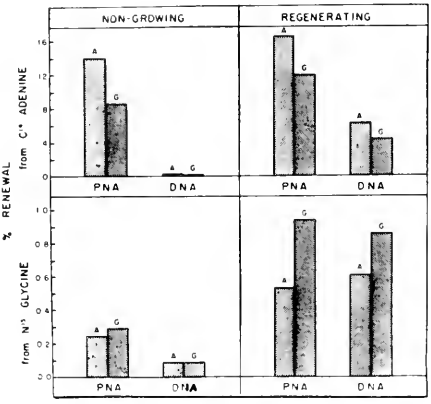

FIG. 3. Per cent of liver PNA and DNA purines derived from each precursor after simultaneous administration of adenine and glycine. (Note that the scale is 20 times larger in the lower section.) Furst, *et al.*[119]

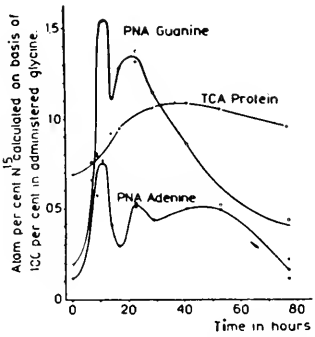

FIG. 4. Isotope contents at different stages of regeneration in rat liver. Glycine-N^{15} injected at various times after operation and animals sacrificed eight hours later. Hammarsten.[127]

shows several distinct differences between the renewals of individual bases in each of two fractions, but cannot account for a continuous synthesis of some small portion of the DNA while the bulk remains static.

In the one instance where the simultaneous incorporations of two precursors into the same moieties were studied in both normal and in regenerating liver,[119] the *relative differences* between the incorporations of glycine and adenine into the PNA and DNA purines of the nongrowing and rapidly growing livers were different, and suggests that if two mechanisms are involved they do not function to the same relative extents in the nongrowing and the rapidly growing tissues. These results (Fig. 3) indicated that in the DNA of the rapidly growing livers the incorporation of glycine nitrogen was 6- to 9-fold greater than into the DNA of the nongrowing, while with adenine there was a 25- to 32-fold difference between the growing and nongrowing livers. In the case of the PNA there was a much greater incorporation of the glycine in the regenerating liver but there was only a small difference in the extent of incorporation of adenine into the PNA under the two conditions. Anderson and Åqvist[124] find that incorporations of orotic acid-N^{15} and phosphate-P^{32} into the pyrimidines and the phosphate also bear different relations to one another in normal and in regenerating liver (Table V, lines 113–116).

A series of studies of the parallelism of the incorporations into protein and into polynucleotides of glycine *administered at various time intervals* after the start of regeneration of rat liver introduced the additional complication of the stage of regeneration of the livers. An impressively complex curve (Fig. 4) with multiple maxima for the incorporations into the purines was recorded.[127] No explanation for the nature of the curves was offered and none yet seems apparent, but these curves are an outstanding example of the complexity which can be encountered in isotope incorporation studies.

Final decisions regarding the apparent existence of two mechanisms of synthesis, and of their characteristics, must await further information re-

garding the intermediates on the pathways leading to the immediate polynucleotide precursors. Most studies have involved liver tissue which has a high degree of polyploidy (Chapter 19). The character of the "extra" DNA in those cells of unusually high DNA content and of the processes which lead to that doubling of the DNA may bear on these questions, but is a subject which has not been investigated. It is of interest that the incorporation of formate into kidney, a tissue showing minimal polyploidy, shows[99] the highest PNA:DNA ratio of any organ, and the greatest apparent retention of the isotope once incorporated into the DNA (Tables II and VI). The details of the picture of the correlation of DNA synthesis with mitosis are obscured in the intestine because of the continuous production of, and physical loss of, cells; in the lymphoid tissues for similar reasons; and in the testes because of the slow process of reductive division.

IV. Factors Influencing Polynucleotide Biosynthesis

The proper functioning of any biological system requires that cellular processes be subject to many controls. Although the processes of nucleic acid biosynthesis are undoubtedly affected by conditions such as the nutritional state of the animals, hormone concentrations, and certain physiological stresses, only a few compounds are known which have any apparent direct function in the biosynthesis of nucleic acids and even in these cases the mechanisms of the involvement of the agents in the synthetic processes are not known. Many situations have been described in which the administration of a compound and the induction of a certain physiological state in an animal, is followed by alterations in the amount or in the renewal of tissue nucleic acids. While such conditions can truthfully be described as affecting nucleic acid synthesis, it does not follow that they are directly implicated in biosynthetic schemes. For example, agents which cause hyperplasia will also give rise to an increased rate of nucleic acid synthesis. Yet such agents are not necessarily directly involved in the biosynthesis of nucleic acids but may act only as general stimulants of cellular division. In most cases it is impossible to determine whether the increased nucleic acid production is a cause or an effect of increased mitotic activity. Thus it has been reported[136] that the administration of progesterone to castrated rabbits results in an increased synthesis of the nucleic acids of the endometrium. However, as this compound is known to cause extensive proliferation of the endometrium, it is impossible to attribute to the hormone a direct influence on nucleic acid synthesis.

Similarly, the induction of cellular hypertrophy is often associated with increased PNA content of the cell. An agent which produces hypertrophy might be considered as a stimulant of PNA synthesis, yet in most cases it would be difficult to establish a direct cause-and-effect relationship between

[136] U. Borell, *Acta Endocrinol.* **9**, 141 (1952).

the agent and the increased synthesis. Nevertheless, a number of experiments have been reported in which experimentally created conditions have resulted in alterations of a "normal" pattern of nucleic acid synthesis.

Two methods have been used most frequently to assay the effect of an agent upon the biosynthesis of nucleic acids. In one the differences between the amounts of nucleic acids in the tissues of experimental and control animals are determined. In some investigations the amounts of nucleic acid per unit weight of tissue are determined, but such changes are not necessarily a sufficient indication of a modification of nucleic acid synthesis since they can arise from an alteration in amounts of the other cellular constituents alone. A change in the nucleic acid content per cell is a much more reliable indication of an alteration of nucleic acid synthesis. On the basis of the widely accepted hypothesis of a constant DNA content per nucleus (Chapter 19), an observation of an altered PNA:DNA ratio is a good indication of a change in PNA synthesis. In the other method differences in the extent of incorporation of labeled compounds into the nucleic acids of experimental and control animals are determined. If a difference is observed, it can be assumed that the agent had an effect, either direct or indirect, upon nucleic acid biosynthesis.

1. Role of Vitamins

a. Folic Acid

It has been demonstrated in a number of microorganisms that folic acid and *p*-aminobenzoic acid are involved in the introduction of carbons 2 and 8 in the purines and the methyl group of thymine[137] (Chapter 23). In mammals, folic acid is one of the few agents which has been demonstrated to have a specific function in nucleic acid synthesis. Drysdale *et al.*[52] found, in rats made deficient in folic acid by means of a diet containing succinylsulfathiazole, that there was a considerably lower incorporation of formate-C^{14} into the purines of liver nucleic acid than into the corresponding purines of rats made deficient and then treated with folic acid. No difference was observed in the extent of incorporation of formate into the purines of the remaining viscera in the deficient and the treated animals.

Skipper *et al.*[138] found that, following the treatment of mice with either of the folic acid antagonists, aminopterin or A-methopterin, there was a 14-fold decrease in incorporation of formate-C^{14} into the visceral nucleic acids but only a 0.25-fold decrease of incorporation into the total visceral homogenate, which indicates that the inhibitors were definitely affecting

[137] W. Shive, *Vitamins and Hormones* **9**, 75 (1951).

[138] H. E. Skipper, J. H. Mitchell, Jr., and L. L. Bennett, Jr., *Cancer Research* **10**, 510 (1950).

nucleic acid synthesis. It was later demonstrated[139] that A-methopterin inhibition could be partially relieved by treatment of the animals with folic acid. It is interesting that, in mice bearing a strain of leukemia refractory to treatment by A-methopterin, the incorporation of formate into the leukemic cells was increased by A-methopterin although the usual inhibition of incorporation into visceral nucleic acids was observed.[139]

Goldthwait and Bendich[16] investigated the simultaneous incorporation of formate-C^{14} and adenine-N^{15} into the nucleic acids of aminopterin-treated and control rats, and found that the incorporation of formate was depressed by the inhibitor to a much greater extent than was the incorporation of adenine. This effectively demonstrated that the inhibition of nucleic acid synthesis by means of aminopterin is not a result of a specific interference with nucleic acid synthesis per se, but rather that it can be attributed to interference with the incorporation of formate into purines and into thymine.

Lowe and Barnum[140] found that the PNA content of the liver of folic acid-deficient megaloblastic monkeys is lower than that of normal animals but that folic acid therapy resulted in a PNA content higher than normal. They also found that the deficient animals incorporated P^{32} into liver PNA less effectively than did the normal animals and that this defect in incorporation was also amenable to treatment with folic acid.

b. Vitamin B_{12}

Experiments on the growth of microorganisms have indicated that in nucleic acid biosynthesis vitamin B_{12} is concerned with the formation of deoxynucleosides (particularly thymidine)[141-143] although it probably also has other functions as well[144-146] (see Chapter 24). The incorporation of P^{32} into *L. leichmanii* is stimulated by B_{12}.[147] The PNA and DNA contents (but not the amounts per cell) of the livers of vitamin B_{12}-deficient rats are less than those of the livers of normal animals.[148] This deficiency also results in a decreased incorporation of glycine-N^{15} into the nucleic acids (and protein).[148] The administration of vitamin B_{12} to deficient rats was followed by

[139] H. E. Skipper, C. Nolan, M. A. Newton, and L. Simpson, *Cancer Research* **12,** 369 (1952); H. E. Skipper, L. L. Bennett, Jr., and L. W. Law, *ibid.* **12,** 677 (1952).
[140] C. U. Lowe and C. P. Barnum, *Arch. Biochem. and Biophys.* **38,** 335 (1952).
[141] W. Shive, J. M. Ravel, and R. E. Eakin, *J. Am. Chem. Soc.* **70,** 2614 (1948).
[142] L. D. Wright, H. R. Skeggs, and J. W. Huff, *J. Biol. Chem.* **175,** 475 (1948).
[143] E. Kitay, W. S. McNutt, and E. E. Snell, *J. Biol. Chem.* **177,** 993 (1950).
[144] H. R. Skeggs, *J. Cellular Comp. Physiol.* **38,** Suppl. 1, 227 (1951).
[145] F. Weygand, A. Wacker, and F. Wirth, *Z. Naturforsch.* **6b,** 25 (1951).
[146] M. Downing, I. A. Rose, and B. S. Schweigert, *J. Bacteriol.* **64,** 141 (1952).
[147] I. Z. Roberts, R. B. Roberts, and P. H. Abelson, *J. Bacteriol.* **58,** 709 (1949). I. A. Rose and B. S. Schweigert, *Proc. Soc. Exptl. Biol. Med.* **79,** 541 (1952).

an increase in the PNA, DNA, and protein-N content of the liver[149] (see also Chapter 16).

c. Other Vitamins

Biotin deficiency in rats results in a decreased incorporation of $NaHC^{14}O_3$ into visceral nucleic acid adenine and guanine as well as into arginine, aspartic acid, and citric acid.[150] It has been reported that there is a decrease in the DNA content of spleen of pyridoxine-deficient animals.[151] In rabbits deficient in vitamin E there is an increased content, over that found in normal animals, of PNA and DNA in skeletal muscle and of DNA in liver.[152] It is impossible to conclude on the basis of this evidence, however, that the vitamins biotin, B_6, or E are directly implicated in the synthesis of nucleic acids. The rate of PNA synthesis as measured by P^{32} incorporation was not affected by vitamin C-deficiency in monkeys,[140] although it has been claimed[153] that ascorbic acid is involved in DNA formation in slices of guinea pig tissues or rat sarcoma.

d. Other Drugs

In a study of the effect of nitrogen mustard (bis (β-chloroethyl)methylamine hydrochloride) on developing salamander embryos, it was found that the drug causes a cessation of DNA synthesis but allows PNA synthesis to continue normally.[154] The incorporation of formate-C^{14} into the nucleic acids of mouse viscera is inhibited by treatment of the animal with 2,6-diaminopurine, 8-azaguanine, cortisone, potassium arsenite, urethan, and nitrogen mustard.[155] In the case of the nitrogen mustard, urethan, and 2,6-diaminopurine, this inhibition cannot be due to a general decline in cellular activity since these compounds cause an increase in the overall incorporation of formate and $NaHC^{14}O_3$ into the organs of the viscera. Nitrogen mustard specifically decreased the incorporation of both formate and adenine into liver DNA purines (with little effect on that into PNA).[156] It is not known whether nitrogen mustard has any specific effect on DNA synthesis or whether it inhibits mitosis in some unknown manner.

[149] M. R. Sahasrabudhe and M. V. Lakshminarayan Rao, *Nature* **168,** 605 (1951).
[150] P. R. MacLeod and H. A. Lardy, *J. Biol. Chem.* **179,** 733 (1949).
[151] L. R. Cerecedo, M. E. Lombardo, D. V. N. Reddy, and J. J. Travers, *Proc. Soc. Exptl. Biol. Med.* **80,** 648 (1952).
[152] J. M. Young and J. S. Dinning, *J. Biol. Chem.* **193,** 743 (1951).
[153] B. I. Gol'dshtein, L. G. Kondrat'eva, and V. V. Gerasimova, *Biokhimiya* **17,** 354 (1952); cf. *Chem. Abstr.* **47,** 718 (1953).
[154] D. Bodenstein and A. A. Kondritzer, *J. Exptl. Zool.* **107,** 109 (1948).
[155] H. E. Skipper, J. H. Mitchell, Jr., L. L. Bennett, Jr., M. A. Newton, L. Simpson, and M. Eidson, *Cancer Research* **11,** 145 (1951).
[156] D. A. Goldthwait, *Proc. Soc. Exptl. Biol. Med.* **80,** 503 (1952).

2. Role of Hormones

It has not been demonstrated that any hormone functions specifically in the control of reactions leading to nucleic acid production (see Chapter 16). A small amount of evidence has been accumulated, however, which indicates that hormones may influence nucleic acid biosynthesis.

An increase in the amount of PNA in the uterus and vagina of castrated mice[157] and in the uterus of castrated rats[158] is induced by injections of estradiol. In both of these experiments these changes were associated with increased PNA:DNA ratios, demonstrating that the treatment with the hormone resulted in an increase of PNA per cell. It was also found that the nucleic acid content and the rate of incorporation of P^{32} into the nucleic acids of the uterus of castrated rabbits was increased by treatment with estradiol.[136] In these experiments it was variously noted that the estrogen also caused increases in the amounts of protein, phospholipid, and enzymes, so that it is impossible to assign to estradiol a specific role in nucleic acid synthesis.

It has already been stated that progesterone causes an increase in the rate of incorporation of P^{32} into the nucleic acids of castrated rabbits and it also causes an increase in the nucleic acid content of the endometrium.[136] In the mucosa of the gizzards of pigeons treated with prolactin, there is an increased synthesis of PNA as well as an increased protein synthesis.[157] In all of the above experiments, the changes in nucleic acids induced by hormones have been accompanied by changes in other cellular constituents. However, in a study of the growth of chick heart explants, it was observed that in insulin-treated cultures the PNA content per cell was higher than in untreated cultures, whereas there was no significant difference between treated and untreated cultures in respect to lipid and protein content per cell,[159] which would suggest that insulin may have a specific effect on nucleic acid biosynthesis.

In the animal the biosynthesis of nucleic acids can be influenced by other factors such as irradiation, diet, pregnancy, and the presence of tumors, and these are discussed in Chapters 16 and 26.

V. Present Possibilities for Pathways of Assembly of Polynucleotides

In considering the mechanisms which may be involved in the biosynthesis of the polynucleotides, it must be recognized that our knowledge is very fragmentary indeed. Discussion at this time can at best serve only as a

[157] R. Jeener, *Biochim. et Biophys. Acta* **2,** 439 (1948).
[158] M. A. Telfer, *Arch. Biochem. and Biophys.* **44,** 111 (1953).
[159] I. Leslie and J. N. Davidson, *Biochem. J.* **49,** xli (1951).

guide to those areas where there are great gaps in our knowledge. As the acquisition of further basic information closes some of those gaps, it may be possible, at some future date, to scrutinize, reinterpret, and integrate the present information into a more satisfactory overall picture.

1. Relation of Synthesis *de novo* to Incorporation of Larger Precursors

The developments in the area of the biosynthesis *de novo* of purine derivatives have shown that the completion of the purine ring is accomplished only after attachment of ribose and phosphate (Chapter 23). In all of those studies inosinic acid has been found to be the first complete purine derivative formed,[160,161] and the gap from inosinic acid to "active" adenine or guanine derivatives which can serve as precursors of the polynucleotide purines remains unclosed. The possiblity also remains that the large production of inosinic acid in the widely studied pigeon liver system,[162-166] is a branch off the main pathway which is directed toward nitrogen disposal, and that inosinic acid is not a member of the direct pathway leading to the polynucleotide purines.

The existence of mechanisms for the incorporation of exogenous adenine into polynucleotide guanine and for the incorporation of exogenous guanine into polynucleotide adenine are each demonstrated in various species. In most instances both mechanisms clearly exist, but the conversion in one or the other direction predominates (Fig. 2). Even in the rat, where the conversion of adenine or adenine derivatives into polynucleotide guanine is strongly favored, polynucleotide guanine can arise by pathways other than via adenine derivatives, as shown by the fact that in some instances there is a greater incorporation of small precursors into the guanine than into the adenine.[109] It seems plausible that there is a common metabolic pathway for the assembly of the purine nucleotide skeleton, and that this branches into two pathways leading, respectively, to adenine and guanine derivatives. These general relationships can be schematically depicted as in Fig. 5.

a. The Directness of the Incorporation of Exogenous Adenine

When exogenous adenine or guanine is available, each appears to be incorporated quite directly into "active" derivatives which represent later stages in the respective pathways leading to the polynucleotide purines.

[160] J. M. Buchanan and D. W. Wilson, *Federation Proc.* **12,** 646 (1953).
[161] G. R. Greenberg, *Federation Proc.* **12,** 651 (1953).
[162] W. Schuler and W. Reindel, *Z. physiol. Chem.* **221,** 209, 232 (1933).
[163] N. L. Edson, H. A. Krebs, and A. Model, *Biochem. J.* **30,** 1380 (1936).
[164] Å. Örström, M. Örström, and H. A. Krebs, *Biochem. J.* **33,** 990 (1939).
[165] G. R. Greenberg, *J. Biol. Chem.* **190,** 611 (1951).
[166] M. P. Schulman, J. C. Sonne, and J. M. Buchanan, *J. Biol. Chem.* **196,** 499 (1952).

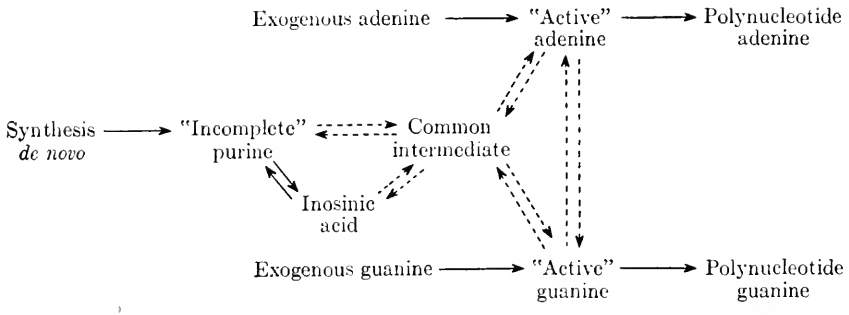

FIG. 5. Possible relationships of purines arising *de novo*, of exogenous purines, and of the interconversion of the purines.

This is borne out by the low dilution factors which can be encountered. From the available estimates of the half-time of the average PNA of the liver of about 8 days, determined by the retention of the isotope incorporated from adenine[17] or from formate,[99] a daily renewal of about 8 to 9% per day is calculated, and it is obvious that the observed renewals of liver PNA adenine of 12% in 36 hours,[15] and of over 22% in 5 days,[17,119] represent the derivation of most of the adenine of the newly synthesized nucleic acids from the administered adenine. Low dilution factors are also encountered with orotic acid and with some nucleosides and nucleotides.

b. Interconversions of the Purines

These could be accounted for by reversal of the reactions leading back to a common intermediate at the point of bifurcation of the pathways (Fig. 5). The alternative of a metabolic bridge between the pathways for a more direct interconversion of adenine and guanine derivatives may not be required. However, its postulation offers certain advantages since there are suggestions that in the course of normal synthesis *de novo* at least a part of either purine may arise via the other, and this cannot be rationalized if newly formed adenine must return through the common intermediate en route to guanine.

The inference that part of either purine may arise via the other is derived from several observations. The relative incorporation of formate into adenine and guanine (the A/G ratio) is generally greater at shorter times (cf. 1-day and 3-day values, Table II, columns 11 and 12). In *L. casei*[40] such a tendency also appears under certain conditions.

In the rat, with glycine-2-C^{14} [118] the A/G ratio was also considerably greater than 1, although with glycine-1-C^{14} [15,123] it was almost exactly 1. With glycine-N^{15} the A/G ratio is always [101,167] less than 1, due partly to the rather specific incorporation of glycine-N into the 2-amino group of guanine; however[109] in some instances (but not always), the purine ring of the guanine may be more extensively labeled than that of adenine (Table IV), lines 4 and 5). On the other hand, with rat liver cell suspensions *in vitro* the incorporation of glycine-2-C^{14} led to an A/G ratio less than 1,[168]

[167] R. Abrams, E. Hammarsten, and D. Shemin, *J. Biol. Chem.* **173,** 429 (1948).
[168] G. A. LePage, *Cancer Research* **13,** 178 (1953).

although with rat liver slices[169] glycine-N^{15} yielded ratios definitely greater than 1, which indicates differences between these systems and the intact animal.

The relative independence of the two pathways in the rat is illustrated by the influence of exogenous adenine in "sparing" *de novo* adenine from glycine[15] or formate[16] to a somewhat greater extent than *de novo* guanine (cf. Table IV, lines 7 *vs.* 6, particularly the values for liver, and Table II, column 8 *vs.* columns 9 and 10). Also, when synthesis *de novo* was largely abolished by a folic acid antagonist, the relative amount of guanine derived from exogenous adenine was considerably increased.[16] If a compound is incorporated into one of the pathways after a point of bifurcation, the extent to which it reaches the other pathway is dependent upon the reversibility of steps back to some intermediate common to a bridge pathway, and this reversibility may vary with physiological conditions of the tissues and the relative demand for the products of each pathway.

A derivative formed from 2,6-diaminopurine must be, or be readily converted into, a member of either the pathway between the common intermediate and the "active" guanine (or of the bridge pathway between the "active" derivatives); the incorporation of diaminopurine into a phosphorylated derivative of 2,6-diaminopurine riboside has been observed in the mouse.[170] 4-Amino-5-imidazolecarboxamide[46] may be incorporated earlier in the assembly line. Certain exogenous purine nucleosides[21] and nucleotides[28,31] can also be converted into the "active" derivatives.

c. Pyrimidine Derivatives

The pyrimidine derivatives require a scheme similar to that in Fig. 5, although the problem of interconversion is the simpler one of amination or deamination. Provision must be made for the incorporation of orotic acid,[57] including its ready conversion into soluble uridine-5′-phosphate derivatives,[171] and for a direct incorporation of each nucleoside and nucleotide.[24,28,64]

Little can be said with certainty regarding pathways of biosynthesis peculiar to DNA. Evidence can be cited to support most of the logical possibilities: a direct deoxygenation of a ribose to a deoxyribose derivative,[64,65] and for an independent origin of deoxyribose[172-174] (Chapter 22) or of de-

[169] P. Reichard and S. Bergström, *Acta Chem. Scand.* **5**, 190 (1951).
[170] G. P. Wheeler and H. E. Skipper, *Federation Proc.* **12**, 289 (1953).
[171] R. B. Hurlbert, *Federation Proc.* **12**, 222 (1953).
[172] E. Racker, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 1, p. 145. Johns Hopkins Press, Baltimore, 1951.
[173] S. S. Cohen, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 1, p. 148. Johns Hopkins Press, Baltimore, 1951.
[174] M. G. McGeown and F. H. Malpress, *Nature* **170**, 575 (1952).

oxyribosyl derivatives.[175] Interchanges are possible between individual deoxyribosyl derivatives[176,177] by transglycosidation mechanisms.

2. The Alternative Metabolic Fates of Administered Compounds

The majority of compounds investigated in attempts to elucidate the character of the larger intermediates involved in the assembly of polynucleotides have perforce been breakdown products of the polynucleotides, or closely related compounds. In many cases these are not normally present in the animal, or at least are of necessity administered in unphysiological quantities. The extent of anabolism, if any, of a given base or its nucleoside or nucleotide into a derivative which is on a pathway leading to polynucleotides will depend upon the balance between its susceptiblity to catabolic enzymes and the rapidity of its anabolism.

a. Correlations with Known Mammalian Enzymes

It is probably premature to attempt to relate the existence of metabolic transformations in intact organisms to the presence of known enzymes. However, despite duplication of material discussed in greater detail in Chapters 15 and 24, a few correlations seem of interest.

The scarcity of tissue pyrimidine nucleosidases (Chapter 15) correlates with the utilization of pyrimidine ribosyl derivatives.[28,64,65]

In the rat the deficiency of adenase[178-180] and the slow action of xanthine oxidase on adenine[181] permit adenine to survive[182] for anabolic fates, while the abundance of guanase and of xanthine oxidase correlates with the preferential catabolism to allantoin of guanine,[12,34] hypoxanthine, and xanthine.[42] 2,6-Diaminopurine is also not deaminated by an extract of rat liver acetone powder.[180] The failure to detect the formation of free adenine (by "trapping" it as 2,8-dioxyadenine) after adenosine-8-C^{14} (or inosine) administration[21] is also in harmony with the lack of enzymes which would be expected to yield adenine from adenosine.

With the purine nucleosides the preferential catabolism of guanosine[21] can be attributed to the presence of nucleoside phosphorylase (Chapters 15 and 24), but the survival of some exogenous inosine for anabolic fates might be explained by assuming a balance in favor of anabolism. The partial catabolism of adenosine and of 2,6-diaminopurine riboside[183] by adenosine deaminase (Chapter 15) might be correlated with the fact that they are less extensively anabolized than the corresponding pu-

[175] R. Ben-Ishai, E. D. Bergmann, and B. E. Volcani, *Nature* **168**, 1124 (1951).
[176] M. Friedkin and H. M. Kalckar, *J. Biol. Chem.* **184**, 437 (1950).
[177] W. S. McNutt, *Biochem. J.* **50**, 384 (1952).
[178] E. J. Conway and R. Cooke, *Biochem J.* **33**, 457 (1939).
[179] D. A. Richert and W. W. Westerfeld, *J. Biol. Chem.* **184**, 203 (1950).
[180] J. Kream and E. Chargaff, *J. Am. Chem. Soc.* **74**, 4274 (1952).
[181] H. Klenow, *Biochem. J.* **50**, 404 (1952).
[182] F. S. Philips, J. B. Thiersch, and A. Bendich, *J. Pharmacol. Exptl. Therap.* **104**, 20 (1952).
[183] D. A. Clarke, J. Davoll, F. S. Philips, and G. B. Brown, *J. Pharmacol. Exptl. Therap.* **106**, 291 (1952).

rines. However, since adenosine is more extensively anabolized than is inosine (in both the rat[21] and in *L. casei*[32]), there must be some direct anabolism of it. Adenosine phosphokinase[30,184] will bring about a phosphorylation of adenosine or of 2,6-diaminopurine riboside in the 5′-position, and offers a possible anabolic route for those nucleosides.

The question of whether the nucleoside phosphorylase (Chapter 24) normally plays a synthetic role remains unanswered. In *in vitro* studies it has been demonstrated that this equilibrium may be far toward the synthetic side. This enzyme is effective with the nucleosides of hypoxanthine or guanine, the purines of which are not utilized by the rat (although guanine can be utilized by many species), but it has not been demonstrated to effect adenosine. Kalckar[74] has pointed out that, if purine nucleoside phosphorylase is capable of catalyzing a transfer of ribose from ribose-1-phosphate to adenine or to 2,6-diaminopurine, the detection of such a reaction would be difficult if the equilibrium were much less favorable than that with hypoxanthine or guanine. It was reported[185] that calf liver extracts are able to catalyze the formation of a purine nucleotide from ribose phosphate (from yeast adenylic acid) and adenine, guanine, or hypoxanthine, but not from xanthine. Consideration of the possibility of an incorporation in the mammal of purine nucleosides via a type of transglycosidation[74] with nucleotides must await information as to whether the ribose of purine nucleosides behaves like that of cytidine[65] and accompanies the purine into polynucleotides.

An indication of some lack of specificity of some of the enzymes dealing with the anabolism of nucleic acid components is suggested by the incorporation of 5-bromouracil into the polynucleotides of *Streptococcus faecalis*[186] and *Enterococcus*,[187] and that of azaguanine into mouse,[188] *T. gelcii*[189] or plant virus nucleic acids,[190] the latter case including a definite demonstration of its incorporation into glycosidic linkage. 2,6-Diaminopurine has been demonstrated to be incorporated into an acid-soluble phosphate of 2,6-diaminopurine riboside.[170]

3. Polynucleotide Synthesis in *L. casei*

In this microorganism, an overall pattern of the utilization of many labeled purine derivatives has been elaborated.[32,33,40,80,81] There is no evidence of degradation of purines by this organism,[40] and the total quantity of the purine consumed during growth can approximate that found in the cells produced, so the use of the growing microorganism presents a system in which the results represent almost exclusively the synthetic phase of nucleic acid metabolism.

Under conditions which will allow synthesis *de novo* and where the added purine does not further stimulate growth (in media containing folic acid),

[184] R. Caputto, *J. Biol. Chem.* **189,** 801 (1951).
[185] J. Wajzer and F. Baron, *Bull. soc. chim. biol.* **31,** 750 (1949).
[186] F. Weygand, A. Wacker and H. Dellweg, *Z. Naturforsch.* **7b,** 19 (1952).
[187] F. Weygand and A. Wacker, *Z. Naturforsch.* **7b,** 26 (1952).
[188] J. H. Mitchell, Jr., H. E. Skipper, and L. L. Bennett, Jr., *Cancer Research* **10,** 647 (1950).
[189] M. R. Heinrich, V. C. Dewey, R. E. Parks, Jr., and G. W. Kidder, *J. Biol. Chem.* **197,** 199 (1952).
[190] R. E. F. Matthews, *Nature* **171,** 1065 (1953).

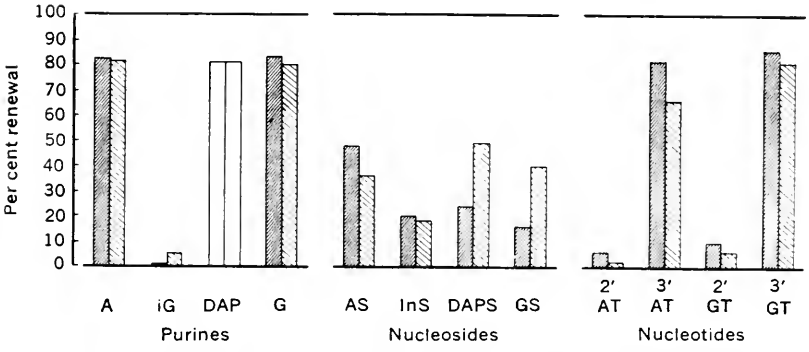

FIG. 6. Utilization of purines and purine derivatives by *L. casei*.

The incorporation of equimolecular amounts of supplements added to a medium containing 0.05 mμg. of folic acid per ml. The left column of each pair represents the renewal of the adenine, the right the renewal of the quanine.

A, adenine; G, guanine; iG, isoguanine; DAP, 2,6-diaminopurine; and an added S refers to the riboside (InS, inosine), and an added T to the ribotide.

there is a strong preferential utilization of preformed purines with a consequent suppression of the synthesis *de novo*.[40,85] In experiments where only the purine moieties were labeled, the relative capabilities for utilization of a series of purine derivatives in competition with the synthesis *de novo* were compared.[32,33,40] In some instances the synthesis *de novo* was also measured directly in replicate experiments with labeled formate or glycine.[40,81]

In Fig. 6 the pairs of columns represent the adenine and guanine of the PNA, and the extent of conversion of the supplement into each purine is indicated. Under these conditions adenine or guanine (also hypoxanthine or xanthine) furnishes about 80% of the PNA purines. 2,6-Diaminopurine alone inhibits all growth in this medium[191] but when present with adenine or with guanine it is utilized as readily as are those purines,[40] and the open columns in Fig. 6 are to indicate that ability to utilize diaminopurine.

The purine moieties of the nucleosides are considerably less extensively utilized[32] than are the free purines. Adenosine was converted preferentially into PNA adenine, and guanosine and diaminopurine riboside were converted preferentially into PNA guanine, facts which are in accord with an assumption that they are initially transformed by substitution or exchange reactions into the respective "active" derivatives on the pathways leading to polynucleotide purines (Fig. 5). Inosine was less efficiently utilized than the above three, and the incorporation into the two PNA purines was essentially equal, which is in harmony with the expectation that it is incorporated prior to the bifurcation of the pathways. The facts that 2,6-diaminopurine riboside is utilized under conditions where its parent purine

[191] G. B. Elion and G. H. Hitchings, *J. Biol. Chem.* **187**, 511 (1950).

is not, and that the interconversions of the ribosyl purines are different from the corresponding free bases, suggest that utilization of nucleosides is not via the free bases. Also, since the nucleosides are utilized less effectively than the free bases (which is also true for xanthosine[32]), the free bases are probably not first converted to the ribosides.

In the *L. casei* there was an extreme difference between the utilization of the purine moieties of the isomeric purine nucleotides. The incorporations[33] of the 3′-isomers were essentially identical with those of the corresponding free purines and were far greater than those of the nucleosides. In contrast, there was no significant utilization of the 2′-isomers. The 3′-isomers of the nucleotide of either purine also support growth (in folic acid-free medium) as effectively as do the purines, while the 2′-isomers and adenosine-5′-phosphate are very much less effective.[33] A specific response to the 3′-isomers of nucleotides is also shown by several purine-requiring mutants of *E. coli* and *Bacillus subtilis*.

The facts that adenosine-3′-phosphate is not effective in reversing the inhibitory effects of diaminopurine[33] although adenine does effect that reversal, and that adenosine-3′-phosphate is efficiently utilized by a diaminopurine-resistant mutant which does not utilize adenine readily,[81] also suggest that the nucleotide is not metabolized via the free purine. The extent of utilization of the 3′-nucleotides is compatible with an assumption that the 3′-nucleotides could be one of the first products formed from the free purines in this species. However, the contrast with the response of the rat, which utilizes the 2′- and 3′-isomers of adenylic acid to equal extents,[31] makes any generalization impossible. The poor utilization of adenosine-5′-phosphate for growth suggests that that compound may not be the "active" polynucleotide precursor.

The pertinence of the presence in *L. casei* of a specific 3′-nucleotidase liberating inorganic phosphate[192] is not clear since there is no evidence of a degradation of the nucleotides in the course of their incorporation. The incorporation by *L. leichmanii*[65] of the ribose as well as the purine of a guanylic acid (isomerism not specified) is in accord with the evidence for direct utilization of nucleotides by *L. casei*;[33] however, the cleavage of cytidine during its utilization by *E. coli*, but not by the rat,[65] again suggests restraint in interpretation.

The question of differential permeability is occasionally raised in attempts to explain seemingly anomalous utilizations, but is frequently unapproachable experimentally. However, in the cases of the utilization of the 3′-nucleotides by *L. casei*, of guanylic acid by *L. leichmanii*,[65] and of certain dinucleotides by *L. helveticus*,[193,194] the factor of permeability would seem to be excluded.

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[192] L. Shuster and N. O. Kaplan, *J. Biol. Chem.* **201**, 535 (1953).
[193] R. B. Merrifield and M. S. Dunn, *J. Biol. Chem.* **186**, 331 (1950).
[194] R. B. Merrifield and D. W. Woolley, *J. Biol. Chem.* **197**, 521 (1952).

4. The Synthesis of Virus Nucleic Acids

With certain bacteriophages the maintenance of the physical integrity of the virus particle after lysis of the host cell permits the isolation of uniform and reproducible samples of newly synthesized nucleoprotein, and many of the known polynucleotide precursors have been applied in studies of virus reproduction, which is discussed in detail in Chapter 26. Virus multiplication obviously involves a distortion of the normal metabolic characteristics of the host, and alterations of enzyme[195,196] and metabolic patterns[50,197-201] of infected bacteria or tissues have been demonstrated. Even though the metabolic machinery of the host is reoriented in virus replication, the processes are probably not so basically altered but that studies of the origin of bacteriophage represent an important facet of the general picture of nucleic acid biosynthesis.

The synthesis of the viral DNA appears (Chapter 26) to involve the assembly of relatively small units which may be obtained from suitable available sources (medium, host, or infecting particles) or by synthesis *de novo*. These units certainly include intact purines, pyrimidines, and polynucleotide phosphorus, and circumstantial evidence also suggests that mono- or possibly oligonucleotides may be involved. There can be extensive utilization of moieties from the DNA of the host, but if there is any transfer of specific nucleic acid fragments of considerable size, from either the infecting particles or the host, it has been masked by the nonspecific transfer which occurs.

5. The Question of the Character of the "Active" Derivatives of the Purines and Pyrimidines

The evidence from the intact animal, from microorganisms, and from viral replication thus suggests a participation of phosphorylated derivatives in the assembly of polynucleotides. In addition the presence in the cytoplasm of egg cells of large amounts of deoxyribosyl derivatives[202,203] (presumably of purines[204] and thymine[205]) also suggests that these are stores of

[195] S. Friedman and J. S. Gots, *Federation Proc.* **11**, 216 (1952).
[196] D. J. Bauer, *Brit. J. Exptl. Pathol.* **32**, 7 (1951).
[197] S. S. Cohen, *Bacteriol. Revs.* **15**, 131 (1951).
[198] D. Kay, *Abstr. 2nd Intern. Congr. Biochem., Paris* p. 85 (1952).
[199] M. E. Raphelson, R. J. Winizler, and H. E. Pearson, *J. Biol. Chem.* **193**, 205 (1952).
[200] L. M. Kozloff, *Cold Spring Harbor Symposia Quant. Biol.* **17**, 207 (1953).
[201] S. S. Cohen, *Cold Spring Harbor Symposia Quant. Biol.* **17**, 221 (1953).
[202] E. Hoff-Jørgensen, *Biochem. J.* **50**, 400 (1952).
[203] E. Hoff-Jørgensen and E. Zeuthen, *Nature* **169**, 245 (1952).
[204] M. Steinert, *Arch. intern. physiol.* **60**, 192 (1952).
[205] D. Elson and E. Chargaff, *Experientia* **143**, (1952).

building blocks for the extensive polynucleotide synthesis which is to occur. Evidence as to the possible character of the intermediates actually involved in the later steps of the biosynthetic processes is just beginning to be accumulated.

a. The Constituents of the Acid-soluble Fractions

The abundance of adenosine-5′-phosphates in the acid-soluble fractions of tissues led initially to the tacit assumption[71,123,132,168,206] that tissue AMP or its polyphosphates probably serve as the source of purines, at least of adenine, for polynucleotide synthesis. The ATP of muscle did not receive serious consideration as a polynucleotide precursor since the phosphate attached to its ribose was one of the first biochemical moieties shown to be but slowly renewed,[207,208] and subsequently the same was demonstrated for its nitrogen[11,209] and its adenine.[12,13] After the administration to rats of adenosine-8-C^{14} a similarly low renewal of the muscle ATP was found.[21]

However, after the administration of adenosine,[21] the total acid-soluble adenine from the muscle was 2.5 times as active as the ATP fraction (isolated as the Ba salts according to LePage[210] and containing some ADP). Such a difference in activity was not observed after the administration of inosine-8-C^{14}. The possibility of the presence of a molecule such as the proposed diadenosine tetraphosphate[211,212] in which the two adenine moieties were not renewed at equal rates was considered. The findings[31] that the 2′- and 3′-phosphates of adenosine are incorporated into muscle adenosine polyphosphates (separated chromatographically according to Cohn[213]) several times as extensively as is the 5′-isomer also fail to support a hypothesis that muscle ATP arises simply by further phosphorylation of AMP.

In contrast to the adenine derivatives in muscle, other soluble adenine derivatives are rapidly renewed. The acid-soluble adenine of yeast[61] or of rat liver cells[168] was extensively renewed by glycine. Exogenous adenine-8-C^{14} was shown to be considerably more rapidly incorporated into the AMP, ADP, and an ATP of rat liver and other viscera (Marrian[214]), and a similar result was obtained in the mouse by Bennett.[215] Simultaneously Goldwasser[216,217] demonstrated that these nucleotides were derived from adenine

[206] J. M. Buchanan, *J. Cellular & Comp. Physiol.* **38,** Suppl. 1, 143 (1951).
[207] T. Korzybski and J. K. Parnas, *Bull. soc. chim. biol.* **21,** 713 (1939).
[208] H. M. Kalckar, J. Dehlinger, and A. Mahler, *J. Biol. Chem.* **154,** 275 (1944).
[209] H. Kalckar and D. Rittenberg, *J. Biol. Chem.* **170,** 455 (1947).
[210] G. A. LePage, *Biochem. Preparations* **1,** 5 (1949).
[211] W. Kiessling and O. Meyerhof, *Biochem. Z.* **296,** 410 (1938).
[212] P. Ohlmeyer, *Federation Proc.* **9,** 210 (1950).
[213] W. E. Cohn and C. E. Carter, *J. Am. Chem. Soc.* **72,** 4273 (1950).
[214] D. H. Marrian, *Biochem. et Biophys. Acta* **9,** 469 (1952); **13,** 282 (1954).
[215] E. L. Bennett, *Abstr. 2nd Intern. Congr. Biochem., Paris* p. 197 (1952).
[216] E. Goldwasser, *Abstr. 2nd Intern. Congr. Biochem., Paris,* p. 200 (1952).
[217] E. Goldwasser, *Nature* **171,** 126 (1953).

in homogenates of pigeon liver, and that an as yet unidentified adenosine phosphate, not identical with AMP, ADP, ATP, or adenosine-3′-phosphate, could contain up to 30% of the administered radioactivity. Goldwasser noted[217] that the amount of his "ATP" fraction as determined enzymatically was about one-half that indicated by the absorption at 260 mμ, and Marrian recorded[214] that his "ATP" fraction contained only a little over one easily hydrolyzable phosphorus. Thus the character of the "ATP" fraction is in question, which is reminiscent of the uncertainty regarding the character of the ATP fractions from plant sources,[218] and of commercial ATP preparations.[219]

The available time curves (Fresco and Marshak[122]) for the incorporation of adenine into the acid-soluble and nPNA fractions in mouse liver indicate that the total acid-soluble adenine does not comply with the requirements for a precursor-product relationship.[106] The activity of the acid-soluble adenine is still rising when that of nPNA has begun to decay, and the acid-soluble adenine does not decay as rapidly as does the nPNA. It would appear[122] that some particular component(s) of the acid-soluble fraction, with an activity higher and with a shorter half-time than that of the fraction as a whole, could be serving as the adenine precursor of the polynucleotides and of other acid-soluble components. That the "active adenine" may be converted into, and may be in equilibrium with, the larger "reservoir" of adenosine-5′-phosphates is suggested by all of these experiments.

The demonstrations[220] that, many days after the administration of adenine, the acid-soluble adenine of an implanted tumor, or that of an initially "unlabeled" parabiotic twin, may acquire a relatively high activity although little incorporation into the polynucleotides is observed also does not suggest that the total acid-soluble adenine represents an "active" polynucleotide precursor. The fact that administered adenosine-5′-phosphate is only about one-half as extensively incorporated into PNA purines[31] as are equimolecular amounts of the 2′- or 3′-isomers cannot of itself be interpreted to indicate that AMP is not closely related to the "active adenine," since it is undoubtedly more extensively diluted and also may be more susceptible to catabolism. However, that behavior also questions the possibility that AMP is in rapid equilibrium with the "active" precursor.

Recent demonstrations of the presence of highly active acid-soluble guanine derivatives derived from glycine in rat liver cell suspensions,[168] and the formation *in vivo* of uridine-5′-phosphates from orotic acid[171] and of a 2,6-diaminopurine riboside phosphate from 2,6-diaminopurine[170] also indicate the rising interest in the metabolic products present in the acid-

[218] H. G. Albaum, M. Ogur, and A. Hirschfeld, *Arch. Biochem.* **27**, 130 (1950).
[219] A. Kornberg, *J. Biol. Chem.* **182**, 779 (1950).
[220] J. Dancis and M. E. Balis, *J. Biol. Chem.* **207**, 367 (1954).

soluble fraction. However, in view of the uncertainty as to the character of the "active adenine," and of the complexity of the acid-soluble nucleotides,[171,217,221] including those of muscle,[21,222,223] decision must be reserved as to the relationship of the currently recognized metabolites in the acid-soluble fractions to the actual "active" polynucleotide precursors.

b. The Possible Positions of Mono- or Oligonucleotides as Polynucleotide Precursors

In the intact animal, purine and pyrimidine nucleotides have, in every case except that of the adenylic acids, been found to be utilized as readily as, or more extensively than, any of the simpler derivatives. Guanylic acid commands particular attention, for in the rat its purine moiety can be utilized far more readily than can any of the simpler derivatives.

The recent observation that the P^{32} of either guanosine-2′- or -3′-phosphate-P^{32} administered to the rat was greatly diluted and was almost uniformly distributed between the individual nucleotides of the PNA[70a] suggests that, despite the virtue of the phosphorylated derivative as a precursor, it may not be incorporated *in toto*. It does seem that the 2′- or 3′-nucleotide is simply a susceptible substrate and that it is further anabolized into the "active" precursor with the eventual loss of the original phosphorus.

It is possible that an individual phosphate introduced into a polynucleotide as part of one nucleotide might, upon alkaline hydrolysis, be removed as part of the nucleotide derived from the adjacent base. However, the present concepts of a grouping of the purines and of the pyrimidines in the polynucleotide structure (Chapter 12) would not suggest that complete randomization of the phosphate could be expected in the course of alkaline hydrolysis. Future experiments involving enzymic degradation of the nucleic acids can possibly avoid that ambiguity.

The ability of the rat to incorporate free adenine more extensively than the 2′-, 3′-, or 5′-nucleotides is also suggestive that none of those nucleotides represent the "active" derivative which arises from the free adenine. From the results in the rat, no particular importance can be attached to any one of the isomeric nucleotides. However, the specific growth responses of several microorganisms to the 3′-isomers, their preferential utilization by *L. casei* under conditions where they are not required for growth (Fig. 6), and particularly the utilization of 3′-adenylic acid by the *L. casei* mutant which utilizes adenine very poorly,[81] suggest that the 3′-phosphates may have some special significance. Such a notion is in harmony with the importance of the 3′-linkage in polynucleotides (Chapter 12). The information from

[221] J. Sacks and L. Lutwak, *Federation Proc.* **12,** 468 (1953).
[222] O. Snellman and B. Gelotte, *Nature* **168,** 461 (1951).
[223] P. A. Khairallah and W. F. H. M. Mommaerts, *Circulation Research* **1,** 8, 12 (1953).

studies of virus reproduction carries the implication that nucleotides or small polynucleotides may be transferred in the assembly of the newly formed DNA. Thus the indications from the several systems studied point to an importance of nucleotides in the final steps of the biosynthesis of the nucleic acids, but the details of the roles of the individual nucleotides and of the character of the "active" precursors must await further experimentation. It has recently been observed[224] that an enzyme-bound AMP is involved in the activation of acetate by coenzyme A, and suggested that such an enzyme-mononucleotide complex may be of more general biosynthetic import.

In this connection some results on the growth response of *L. helveticus* 355 may be pertinent. That organism responds only to uracil, to a partial hydrolysate of PNA,[193] and to a number of dinucleotides which possess a terminal cytidine-5′-phosphate.[194] The requirement does not appear to be for the dinucleotides as such, but the dinucleotides have in common the availabity of the specific terminal group. Conceivably "active" precursors could be derivatives of this type which merely serve as carriers for the transfer of a specific group.

The question[99,123,130] as to whether the biosynthesis of polynucleotides proceeds by a stepwise buildup, unit by unit, or whether there is a simultaneous assembling of all of the components is a repetition of the corresponding debate with regard to protein synthesis. The chemical (Chapter 11) heterogeneity of the PNA preparations yet studied, the inequalities of the incorporations of pyrimidine or purine precursors into different units in a given nucleic acid, and the metabolic[59] and chemical[99,225,226] heterogeneity (Chapter 10) of DNA preparations are all compatible with a stepwise synthesis or a partial renewal. Adequate evidence regarding a simultaneous assembly will have to await availability of characterized homogeneous preparations of polynucleotides.

VI. Addendum

New information on the character of the soluble nucleotides present in tissues is rapidly accumulating. Uridine-5′-triphosphate has been prepared from yeast.[227] A uridine-5′-monophosphate, among others, has been recognized in a TCA extract of liver.[228] The presence of 5′-monophosphates, -diphosphates, and -triphosphates of uracil, cytosine, adenine, and guanine in

[224] Mary E. Jones, F. Lipmann, H. Hilz, and F. Lynen, *J. Am. Chem. Soc.* **75,** 3286 (1953).

[225] E. Chargaff, C. F. Crampton, and R. Lipshitz, *Nature* **172,** 289 (1953).

[226] G. L. Brown and M. Watson, *Nature* **172,** 339 (1953).

[227] S. H. Lipton, S. A. Morell, A. Frieden, and R. M. Bock, *J. Am. Chem. Soc.* **75,** 5449 (1953).

[228] J. Sacks, L. Lutwak, and P. D. Hurley, *J. Am. Chem. Soc.* **76,** 424 (1954).

a single perchloric acid extract of rat tissues has been demonstrated[229] (cf. footnote 171). The separation of an adenosine tetraphosphate (with three labile phosphates) from commercial ATP is reported.[230]

Possible anabolic routes for nucleosides and adenine are suggested by newly recognized enzymatic activities. A preparation from malt diastase will accomplish phosphorylation of ribo- or deoxyribonucleosides by transfer of phosphate from phenylphosphate.[231] The incorporation of adenine directly into a nucleotide (AMP) through the action of ribose-1,5-diphosphate and a pigeon liver nucleotide phosphorylase is described.[232]

Demonstration of some incorporation of glycine and adenine into the DNA of mature sea urchin sperm, *in vitro*, gives direct evidence of the formation of DNA in the absence of mitosis.[233] In brain slices an incorporation of inorganic P^{32} into the PNA nucleotides, but little (and that of doubtful validity) into the DNA, was observed,[234] which is in harmony with the results with formate *in vivo*[99] rather than with the earlier ones with phosphate.[10]

Studies of the incorporation of the 2′- and 3′-phosphates of cytidine have shown that they are equally readily incorporated into the nucleic acids of regenerating rat liver.[235]

Further reports have appeared on the incorporation of purines into *T. gelli*[236] (cf. footnote 83), on the incorporation *in vivo* of 2,6-diaminopurine into a phosphate derivative of its nucleoside[237] (cf. footnote 170), and on the incorporation *in vivo* of adenine into soluble nucleotides.[238] (cf. footnote 215).

[229] H. Schmitz, V. R. Potter, R. B. Hurlbert, and D. M. White, *Cancer Research* **14,** 66 (1954).
[230] D. H. Marrian, *Biochim. et Biophys. Acta* **12,** 492 (1953).
[231] G. Brawerman and E. Chargaff, *J. Am. Chem. Soc.* **75,** 2020 (1953).
[232] M. Saffran and E. Scarano, *Nature* **172,** 949 (1953).
[233] H. M. Malkin, *Biochim. et Biophys. Acta* **12,** 585 (1953).
[234] H. A. Deluca, R. J. Rossiter, and K. P. Strickland, *Biochem. J.* **55,** 193 (1953).
[235] P. Reichard, *Acta Chem. Scand.* **7,** 862 (1953).
[236] M. Flavin and M. Engelman, *J. Biol. Chem.* **200,** 59 (1953).
[237] G. P. Wheeler and H. E. Skipper, *J. Biol. Chem.* **205,** 749 (1953).
[238] E. L. Bennett, *Biochim. et Biophys. Acta* **11,** 487 (1953).

CHAPTER 26

The Metabolism of the Nucleic Acids

R. M. S. SMELLIE

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I. Introduction

Between ribonucleic acid (PNA) and deoxyribonucleic acid (DNA) there is a major difference in metabolic properties; the DNA remains relatively constant in amount throughout the greater part of the life of the cell while the amount of PNA may vary considerably. This suggests that DNA may be a comparatively inert component, while PNA is actively engaged in the metabolic processes of the cell. While this may be true of resting cells, rapid synthesis of DNA must necessarily take place during division if one cell is to give rise to two, each with the same DNA content as the parent (Chapter 19). It may be expected therefore that PNA will prove to be metabolically active even in resting tissues, but that DNA will be relatively inert in resting tissues and more active in proliferating tissues such as intestinal mucosa, bone marrow, and regenerating liver.

The advent within recent years of isotope techniques in biological research has overcome many of the difficulties which confronted earlier workers on the metabolism of the nucleic acids, and the way has been opened to studies on their biosynthesis, metabolism, and catabolism. The aspects of nucleic acid metabolism associated with biosynthesis have been discussed in the preceding chapters of this volume, and this chapter will be restricted as far as possible to a discussion of the metabolic activity and catabolism of the nucleic acids.

II. The Use of P^{32} in Studies on Nucleic Acid Metabolism

1. DNA

a. The Relative Activities of the DNA's from Different Tissues

Radioactive phosphorus has been widely used in studies on the metabolism of the DNA from different tissues. Some of the results obtained are illustrated in Table I (see also Table II in Chapter 25). From these figures, which are expressed as ratios relative to the activity of the spleen DNA in each group, it is evident that the tissues fall into two main classes with respect to incorporation of P^{32} into the DNA: those such as bone marrow, spleen, thymus, and intestinal mucosa, which may be regarded as proliferating tissues, in which uptake of isotope is high; and the remainder

typified by liver, kidney, and brain, in which the isotope content is small and cell division minimal in the normal adult animal. It has been found[1,2] that there is a decrease with age in the rate of formation of labeled DNA in thymus, lymph nodes, and spleen and that the uptake of P^{32} by the DNA of weanling rat liver is more rapid than in the adult animal.[3] This work lends support to the conception that P^{32} incorporation into the DNA of a tissue provides an indication of the rate of cell division in that tissue. Further evidence for this view comes from the work of Hevesy and Ottesen,[4] who were unable to obtain incorporation of radioactive phosphorus into the DNA of nucleated hen erythrocytes (nondividing cells) incubated in the presence of labeled phosphate, and also from the work of Howard and Pelc,[5,6] who have shown by their autoradiograph technique on bean roots that a cell which has completed its last division and is differentiating does not synthesize DNA.

Stevens *et al.*[7] have compared the rate of incorporation of P^{32} into the DNA of rat liver and intestine with the rate at which new cells are formed by mitosis in these tissues. They found that twice as much phosphorus appeared in the DNA as could be accounted for by the synthesis of additional DNA, and suggest that all the DNA derived from the mitosis is newly formed.

b. The Effect of Physiological and Pathological Changes on the Uptake of Radioactive Phosphorus by DNA

The conclusions of the previous section indicate that changes in the physiological or pathological state of a tissue which tend towards increased cell division will lead to increased incorporation of radioactive phosphorus into the DNA of the tissue. The results summarized in Table II make it clear that such indeed is the case. Thus regenerating rat liver, fetal rabbit liver, rat hepatoma, and pregnancy-stimulated mammary gland, as well as carcinomatous mammary gland, all show greatly increased incorporation of isotope as compared with the corresponding normal tissue in each case.[12-16]

[1] E. Andreasen and J. Ottesen, *Acta Physiol. Scand.* **10,** 258 (1945).
[2] G. Hevesy, *Advances in Enzymol.* **7,** 111 (1947).
[3] R. M. S. Smellie, W M. McIndoe, R. Logan, J. N. Davidson, and I. M. Dawson, *Biochem. J.* **54,** 280 (1953).
[4] G. Hevesy and J. Ottesen, *Nature* **156,** 534 (1945).
[5] A. Howard and S. R. Pelc, *Ciba Conf. on Isotopes in Biochem., London* p. 138 (1951).
[6] S. R. Pelc and A. Howard, *Exptl. Cell Research* Suppl. **2,** 269 (1952).
[7] C. E. Stevens, R. Daoust, and C. P. Leblond, *J. Biol. Chem.* **202,** 177 (1953).
[8] G. Hevesy and J. Ottesen, *Acta Physiol. Scand.* **5,** 237 (1943).
[9] E. Hammarsten and G. Hevesy, *Acta Physiol. Scand.* **11,** 335 (1946).
[10] A. H. Payne, L. S. Kelly, and H. B. Jones, *Cancer Research* **12,** 666 (1952).
[11] R. M. S. Smellie, E. R. M. Kay and J. N. Davidson, unpublished results.
[12] A. M. Brues, M. M. Tracy, and W. E. Cohn, *J. Biol. Chem.* **155,** 619 (1944).

TABLE I

THE RELATIVE UPTAKES OF P^{32} BY THE DNA OF DIFFERENT TISSUES EXPRESSED AS A RATIO RELATIVE TO THE VALUE FOR THE SPLEEN IN EACH GROUP AS 10

Species	Tissue	Time after administration, hr.	Relative uptake	Ref.
Rat	Spleen	94	10.0	8
	Intestine		22.9	
	Testes		1.4	
	Muscle		2.0	
	Liver		2.3	
	Kidney		1.1	
	Brain		0.3	
Rat	Spleen	2	10.0	9
	Intestine		13.2	
	Liver		0.5	
Rat	Spleen	3	10.0	1
	Bone marrow		185.7	
	Thymus		85.7	
	Intestinal lymph nodes		18.6	
	Skin lymph nodes		18.6	
Mouse	Spleen	4	10.0	10
	Intestine		5.5	
	Liver		0.3	
Rabbit	Spleen	18	10.0	11
	Appendix		96.5	
	Bone marrow		82.2	
	Thymus		35.2	
	Intestinal mucosa		30.8	
	Liver		3.0	
	Kidney		2.0	
	Brain		1.0	

Similarly, the rate of renewal of weanling rat liver DNA is high in comparison with the normal. It has been shown[3,10,14,17,18] that the presence of a

[13] S. Albert, R. M. Johnson, and M. S. Cohan, *Cancer Research* **11,** 772 (1951).
[14] L. S. Kelly, A. H. Payne, M. R. White, and H. B. Jones, *Cancer Research* **11,** 694 (1951).
[15] A. C. Griffin, L. Cunningham, E. L. Brandt, and D. W. Kupke, *Cancer* **4,** 410 (1951).
[16] E. P. Tyner, C. Heidelberger, and G. A. LePage, *Cancer Research* **13,** 186 (1953).
[17] W. M. McIndoe and J. N. Davidson, *Brit. J. Cancer* **6,** 200 (1952).
[18] A. H. Payne, L. S. Kelly, and M. R. White, *Cancer Research* **12,** 65 (1952).

TABLE II

THE RELATIVE UPTAKES OF P^{32} BY THE DNA OF DIFFERENT TISSUES IN DIFFERENT PHYSIOLOGICAL AND PATHOLOGICAL STATES EXPRESSED AS RATIOS RELATIVE TO THE NORMAL RESTING TISSUES IN EACH GROUP AS 10

Species	Tissue	Time after administration, hr.	Relative uptake	Ref.
Rat	Resting liver	72	10.0	12
	Resting liver	192	19.6	
	Regenerating liver	72	109.8	
	Regenerating liver	312	543.4	
	Hepatoma	72	603.6	
Rabbit	Resting liver	4	10.0	3
	Maternal liver		22.0	
	Fetal liver		119.4	
Rat	Resting liver	4	10.0	3
	Regenerating liver		143.3	
	Weanling liver		46.7	
Mouse	Resting mammary gland	17	10.0	13
	Pregnancy-stimulated mammary gland		142.5	
	Carcinomatous mammary gland		127.5	
Rat	Resting liver	18	10.0	15
	Precancerous liver		67.0	
	Hepatoma		136.8	
Rat	Resting liver	2	10.0	14
	Pregnant liver		24.5	

tumor in one tissue in rats, mice, and fowls or of a growing fetus in mice and rabbits exerts a marked effect on the uptake of P^{32} by the DNA of other tissues. Thus in mice with mammary carcinomata and in pregnant mice and rabbits the isotope uptake by the DNA of liver, spleen, and kidney is very much higher than in the corresponding controls; this effect is not observed in the DNA from the intestine of the host animals, nor does the metabolic activity of the PNA of these tissues appear to be greatly disturbed by the presence of a tumor. These studies have been extended by Payne *et al.*,[19] who, using formate-C^{14} and glycine-2-C^{14}, confirmed that

[19] A. H. Payne, L. S. Kelly, G. Beach, and H. B. Jones, *Cancer Research* **12**, 426 (1952).

the uptake of isotope by the DNA of liver and spleen of tumor-bearing animals was greater than in the controls, and noted that the increment in uptake of P^{32} by the DNA of mouse liver showed an increase with increasing age of the tumor. A further observation bearing on this effect is the finding of Kelly and Jones[20] that the administration of homologous tissue extracts to mice for some days, leads to an increased incorporation of P^{32} into the DNA of certain organs. The metabolism of the nucleic acids of neoplastic tissues has recently been reviewed by Heidelberger.[21]

2. PNA

a. Precautions Necessary in the Study of PNA Metabolism with P^{32}

Two factors complicate attempts to study the metabolism of PNA by means of isotopic phosphorus. The first is that the PNA within the cell cannot be regarded as homogeneous in origin since it is found in the various morphological components of the cell. Moreover, as we shall see later, the PNA of the cell nucleus is metabolically very different from that of the cytoplasm. The second factor relates to the danger of contamination of the PNA with other substances which are more highly radioactive than the PNA itself, and which must now be considered.

In most instances, determination of radioactivity has been made on samples of PNA isolated in a purified form by one or other of the methods described in the literature.[12,22-25] While these methods are satisfactory for most purposes, they suffer from the major disadvantage that recovery may not be quantitative and that a trace of some radioactive contaminant may be present in the final product. In an attempt to overcome the difficulties concerning the combination of quantitative measurement of the nucleic acids in tissues simultaneously with isotope measurements, Davidson *et al.*[26,27] have suggested determining the uptake of P^{32} into the PNA and DNA of a tissue by measurements on the corresponding fractions obtained by the application of a fractionation scheme such as that of Schmidt and Thannhauser,[28] which has been described in Chapter 16. Several workers have attempted to measure the radioactivity of tissue PNA and DNA by

[20] L. S. Kelly and H. B. Jones, *Am. J. Physiol.* **172,** 575 (1953).
[21] C. Heidelberger, *Advances in Cancer Research* **1,** 273 (1953).
[22] J. N. Davidson, S. C. Frazer, and W. C. Hutchison, *Biochem. J.* **49,** 311 (1951).
[23] E. Hammarsten, *Acta Med. Scand.* Suppl. **196,** 634 (1947).
[24] J. N. Davidson and R. M. S. Smellie, *Biochem. J.* **52,** 599 (1952).
[25] E. Volkin and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1516 (1951).
[26] J. N. Davidson, M. Gardner, W. C. Hutchison, W. M. McIndoe, W. H. Raymond, and J. F. Shaw, *Biochem. J.* **44,** xx (1949).
[27] J. N. Davidson, M. Gardner, W. C. Hutchison, W. M. McIndoe, and J. F. Shaw, *Abstr. 1st Intern. Congr. Biochem., Cambridge, Engl.* p. 252 (1949).
[28] G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.* **161,** 83 (1945).

such a technique,[29-34] but the danger of contamination of such fractions with highly active non-nucleic acid phosphorus has been stressed by Barnum *et al.*,[35] Friedkin and Lehninger,[36] Jeener,[37,38] and by Jeener and Szafarz,[39] who like Davidson *et al.*[22,40] have shown that there is no justification for the assumption that the specific activities of the nucleic acid fractions obtained by the Schmidt-Thannhauser technique is a measure of the specific activities of the nucleic acids. In the ribonucleotide fraction, this discrepancy is due to the presence of non-nucleotide phosphorus of very much higher activity than the nucleotide phosphorus itself.[24] Jeener and Szafarz[41] and Szafarz and Paternotte[42] have used paper chromatography of the ribonucleotide fraction in an attempt to rid it of contaminating non-nucleotide phosphorus, but the most satisfactory technique seems to be that of Davidson and Smellie,[24] who have subjected the ribonucleotide fraction to ionophoresis, thus separating its constituent nucleotides and at least six other phosphorus-containing compounds, one of which is inorganic phosphate.

b. The Relative Incorporations of Isotopic Phosphorus into the PNA and DNA of Different Tissues

Generally speaking, the uptake of P^{32} by the PNA of resting tissues is considerably greater than the corresponding value for DNA. This difference is illustrated in Table V of Chapter 25, where the ratio of P^{32} in PNA to that in DNA is less than unity only in tissues such as regenerating or fetal liver where the metabolic activity of the DNA is greatly increased. This table shows that the observed ratios vary considerably from tissue to tissue and also with the different times after administration of the isotope, indicating that the relationship between the PNA and DNA metabolic activities is not constant from tissue to tissue or even within the same

[29] R. M. Campbell and H. W. Kosterlitz, *J. Endocrinol.* **6,** 171 (1949).
[30] B. E. Holmes, *Brit. J. Radiol.* **22,** 487 (1949).
[31] B. E. Holmes, *Abstr. 1st Intern. Biochem., Cambridge, Engl.* p. 263 (1949).
[32] W. Hull and P. L. Kirk, *J. Gen Physiol.* **33,** 325 (1950).
[33] W. Hull and P. L. Kirk, *J. Gen. Physiol.* **33,** 335 (1950).
[34] W. Hull and P. L. Kirk, *J. Gen. Physiol.* **33,** 343 (1950).
[35] C. P. Barnum, C. W. Nash, E. Jennings, O. Nygaard, and H. Vermund, *Arch. Biochem.* **25,** 376 (1950).
[36] M. Friedkin and A. L. Lehninger, *J. Biol. Chem.* **177,** 775 (1949).
[37] R. Jeener, *Nature* **163,** 837 (1949).
[38] R. Jeener, *Bull soc. chim. biol.* **31,** 731 (1949).
[39] R. Jeener and D. Szafarz, *Experientia* **6,** 59 (1950).
[40] J. N. Davidson, *Ciba Conf. on Isotopes in Biochem., London* p. 175 (1951).
[41] R. Jeener and D. Szafarz, *Arch. Biochem.* **26,** 54 (1950).
[42] D. Szafarz and C. Paternotte, *Bull. soc. chim. biol.* **33,** 1518 (1951).

tissue at different time intervals, and that the renewal of the two nucleic acids proceeds at different rates in the different tissues.

c. The Uptake of P^{32} by the Individual Nucleotides Derived from the PNA of Different Tissues

Since the phosphorus of PNA is distributed among its four constituent nucleotides, it is desirable to study the renewal of phosphorus in each nucleotide separately.

Several methods for the separation of mononucleotides by paper chromatography (Chapter 7) have been described.[43-45] None of these is suitable for measurements of radioactivity of the individual nucleotides since in every instance one pair of nucleotides is not completely resolved. Volkin and Carter[46] have separated the mononucleotides derived from PNA and DNA by the ion-exchange technique of Cohn[47] (Chapter 6) and have measured the incorporation of radioactive phosphorus into the nucleotides of PNA and DNA from several samples of liver tissue. Davidson *et al.*[3,17,24,40,48] have applied their ionophoretic technique[49] to the separation of the ribose mononucleotides present in alkaline hydrolysates of PNA extracted from several different liver tissues and tissue fractions. Boulanger *et al.*[50,51] have made use of a chromatographic method in their studies on the kinetics of P^{32} incorporation into the PNA of whole rat liver tissue.

From the results of these workers, it appears that in general the specific activities of the four ribose mononucleotides are similar to one another, adenylic acid usually exhibiting the highest activity and guanylic acid the lowest, while the activities of the pyrimidine nucleotides give intermediate values. It may be of significance that the activities of the four nucleotides are approximately in inverse proportion to their relative molar proportions within the PNA.

d. The Effect of Physiological and Pathological Changes on the Metabolic Activity of PNA

We have seen already that the uptake of P^{32} by DNA in regenerating liver and in hepatoma is considerably raised as compared with the normal.

[43] B. Magasanik, E. Vischer, R. Doniger, D. Elson, and E. Chargaff, *J. Biol. Chem.* **186,** 37 (1950).
[44] C. E. Carter, *J. Am. Chem. Soc.* **72,** 1466 (1950).
[45] R. Markham and J. D. Smith, *Biochem. J.* **49,** 401 (1951).
[46] E. Volkin and C. E. Carter. *J. Am. Chem. Soc.* **73,** 1519 (1951).
[47] W. E. Cohn, *J. Am. Chem. Soc.* **72,** 1471 (1950).
[48] J. N. Davidson, *Bull. soc. chim. biol.* **35,** 49 (1953).
[49] J. N. Davidson and R. M. S. Smellie, *Biochem. J.* **52,** 594 (1952).
[50] P. Boulanger, J. Montreuil, and L. Masse, *Compt. rend.* **234,** 565 (1952).
[51] P. Boulanger and J. Montreuil, *Biochim. et Biophys. Acta* **9,** 619 (1952).

TABLE III

THE INCORPORATION OF P^{32} INTO THE PNA OF VARIOUS TISSUES IN DIFFERENT PHYSIOLOGICAL AND PATHOLOGICAL STATES CALCULATED RELATIVE TO THE NORMAL TISSUE IN EACH GROUP AS 10

Species	Tissue	Time after isotope administration, hr.	Relative uptake	Ref.
Rat	Resting liver	72	10.0	12
	Resting liver	192	22.4	
	Regenerating liver	72	42.1	
	Regenerating liver	312	57.2	
	Hepatoma	72	31.2	
Rat	Resting liver	⅓	10.0	46
	Regenerating liver		30.5	
Mouse	Resting liver	⅓	10.0	46
	Hepatoma		10.8	
Rat	Resting liver	4	10.0[a]	3
	Weanling liver		16.2[a]	
	Regenerating liver		35.5[a]	
Rabbit	Resting liver	4	10.0[a]	3
	Maternal liver		10.6[a]	
	Fetal liver		22.6[a]	
Mouse	Resting liver	4	10.0[a]	18
	Liver with mammary carcinoma		10.8[a]	
Mouse	Resting liver	4	10.0[a]	18
	Liver with sarcoma		11.6[a]	
Mouse	Resting mammary gland	17	10.0	13
	Pregnancy-stimulated mammary gland		17.4	
	Mammary carcinoma		14.7	

[a] Cytoplasmic PNA as distinct from total PNA.

The results shown in Table III illustrate the effects of such factors on the incorporation of isotopic phosphorus into PNA. It will be seen that in liver regenerating after partial hepatectomy, the activity of the PNA is three to four times that in resting liver.[3,12,46] Similarly, in fetal rabbit liver the activity of the PNA is increased about twofold compared with the normal, while a somewhat smaller increase is recorded with weanling rat liver.[3] The incorporation of P^{32} into the PNA of pregnancy-stimulated

mammary gland and mammary carcinoma is appreciably higher than in the normal gland.[13] In contrast to DNA, the phosphorus uptake by the PNA of mouse liver does not appear to be greatly affected by the presence of a neoplasm in other tissues.[13,18]

Recently, Munro *et al.*[52] have studied the effects of dietary changes on the PNA content of rat livers and also on the renewal of the PNA phosphorus. They found that, when the animals were fed a protein-containing diet, an increase in energy intake of 1000 kcal. caused a rise of 20 to 30% in the PNA content of the liver, but that there was no significant change in the proportion of phosphorus atoms replaced in a given time. On the other hand, when the animals were fed a protein-free diet, an increase in energy intake of 1000 kcal. caused only a slight rise (5 to 10%) in the PNA content of the liver, but the rate of replacement of phosphorus atoms was augmented by about 25%. It appears therefore that at each level of protein intake the total number of phosphorus atoms incorporated into the PNA is increased by raising the energy intake, in one case by an increase in the amount of PNA per liver without a change in the percentage of phosphorus atoms incorporated in a given time, and in the other case by an increase in the incorporation rate with a smaller change in the amount of PNA per liver.

III. The Metabolism of the Purine and Pyrimidine Bases of PNA and DNA

1. General

While much useful information concerning the metabolism of the nucleic acids has been obtained from studies with labeled phosphorus, it is always possible, although unlikely in view of current views on nucleic acid structure, that the phosphorus moiety behaves differently from the main skeleton of the molecule. The metabolism of the bases of the nucleic acids is therefore of considerable importance. Recent reviews of this topic are by Christman,[53] Franke,[54] and Brown.[55]

2. Small-Molecule Precursors of the Nucleic Acid Purines (See also Chapter 25).

a. The Source of the Individual Atoms of the Purines

From isotopic studies discussed in Chapter 23, it has been concluded that carbon atoms 2 and 8 of the purine ring are derived from formate or

[52] H. N. Munro, D. J. Naismith, and T. W. Wikramanayake, *Biochem. J.* **54,** 198 (1953).

[53] A. A. Christman, *Physiol. Revs.* **32,** 303 (1952).

[54] W. Franke, *Z. Vitamin-Hormon-u. Fermentforsch.* **5,** 279 (1953).

[55] G. B. Brown, *Ann. Rev. Biochem.* **22,** 141 (1953).

substances giving rise to one-carbon compounds, carbon 4 is derived from the carboxyl group of glycine, carbon 5 from the methylene group of glycine, and carbon 6 from carbon dioxide. It has also been shown that nitrogen atoms 1, 3, and 9 are derived from the amino-nitrogen pool and nitrogen 7 from glycine.

A suitable choice of labeled precursor thus enables the metabolism of the individual purine bases to be studied from several different aspects.

b. Studies with Formate-C^{14}

Several authors have used formate-C^{14} in studies of nucleic acid metabolism. Drochmans *et al.*[56] have shown that, after administration of labeled formate to rats, the activity of the PNA purines from different tissues decreased in the order intestine, kidney, spleen, liver, pancreas, and testis. No differentiation was found between the isomeric forms of adenylic and guanylic acids in any of these tissues, and in all tissues except spleen the activity of the adenylic acid was appreciably greater than that of the guanylic acid. Totter *et al.*[57] have studied the utilization of formate-C^{14} by the mononucleotides of visceral PNA and DNA in rats and chickens. They found that there was appreciable uptake of isotope by the purine nucleotides of both nucleic acids while, as expected, the pyrimidine nucleotides showed little activity. In the rat viscera, the adenylic acids of both PNA and DNA exhibited a considerably higher uptake of isotope than did the corresponding guanylic acids. Similar observations on the higher incorporation of C^{14} from formate into the adenine than into the guanine of liver nucleic acids have been recorded by Goldthwait,[58] Smellie *et al.*,[59] and by Drysdale *et al.*[60]

The uptake of formate-C^{14} by the purines of DNA from different tissues in normal mice and in mice with mammary carcinomata has been the subject of research by Payne *et al.*,[10,19] who have observed that the DNA of spleen and intestine is much more active than that of liver, the relative values being: spleen 10, intestine 9.6 to 9.9, and liver 0.3 to 0.4. These values are similar to those found for the same tissues with P^{32} (Table I).

The relative rates of renewal of the purines of PNA and DNA in different tissues are summarized in Table V of Chapter 25. The activity of PNA in general exceeds that of DNA, and the ratios are similar to, although slightly lower than, those obtained with P^{32}.

[56] P. Drochmans, D. H. Marrian, and G. B. Brown, *Arch. Biochem. and Biophys.* **39,** 310 (1952).

[57] J. R. Totter, E. Volkin, and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1521 (1951).

[58] D. A. Goldthwait, *Proc. Soc. Exptl. Biol. Med.* **80,** 503 (1952).

[59] R. M. S. Smellie, W. M. McIndoe, and J. N. Davidson, *Biochem. et Biophys. Acta* **11,** 559 (1953).

[60] G. R. Drysdale, G. W. E. Plaut, and H. A. Lardy, *J. Biol. Chem.* **193,** 533 (1951).

c. Studies with C^{13}- and C^{14}-labeled Glycine

Since the carbon of formate gives rise to carbon atoms 2 and 8 in the purine ring while the carbons of glycine are incorporated mainly into positions 4 and 5, the renewal of either of these pairs of carbon atoms must involve complete rupture of the ring structure. The pattern of incorporation of C^{13}- or C^{14}-labeled glycine should therefore be similar to that obtaining with formate.

Payne *et al.*[10,19] have examined the incorporation of glycine-2-C^{14} into the DNA of different tissues of normal mice and of mice with mammary carcinomata. Once again in normal animals the DNA of spleen and intestine exhibits a much greater uptake of isotope than that of liver, the relative values being about 10, 12, and 1. The presence of a neoplasm leads to greater uptake of isotope by the DNA purines of liver and spleen, although the effect is not so marked as was found with formate.

Several authors[10,19,61-64] have measured the relative incorporation of C^{14} from glycine into the PNA and DNA of different tissues, using glycine labeled in the carboxyl or methylene groups. The results of some of these analyses are shown in Table V of Chapter 25. As with formate and phosphorus, the PNA renewal at any one time is appreciably greater than that of DNA, and as might be expected there is reasonably good correlation between formate and glycine as precursors.

The kinetics of incorporation of glycine-2-C^{14} and P^{32} into the PNA and DNA of rat liver and tumor have been examined by Tyner *et al.*[16,65] They found that the specific activities of the P^{32} and C^{14} in the purine nucleotides of DNA were similar, whereas with the PNA the specific activities of the P^{32} in the purine nucleotides was appreciably higher than the specific activity of the C^{14} in the same nucleotides.

d. The Replacement of the Purine Nitrogen

The metabolism of the nitrogen atoms of the purines of PNA and DNA may be studied with the aid of N^{15}-labeled ammonia or glycine as precursors of the nitrogen in the ring. Unfortunately, the techniques for the estimation of stable isotopes are less sensitive than those for radioactive isotopes, and it is therefore necessary to administer larger doses of the precursor and to isolate larger quantities of material for assay. The dosage of glycine and ammonium salts which is commonly used is so high that it may have a marked effect on the body pools of these substances. Neverthe-

[61] D. Elwyn and D. B. Sprinson, *J. Am. Chem. Soc.* **72,** 3317 (1950).
[62] G. A. LePage and C. Heidelberger, *J. Biol. Chem.* **188,** 593 (1951).
[63] C. Heidelberger and G. A. LePage, *Proc. Soc. Exptl. Biol. Med.* **76,** 464 (1951).
[64] R. Abrams, *Arch. Biochem. and Biophys.* **33,** 436 (1951).
[65] E. P. Tyner, C. Heidelberger, and G. A. LePage, *Cancer Research* **12,** 158 (1952).

less, much valuable information concerning the metabolism of the nucleic acids has been obtained using N^{15}-labeled precursors.

Experiments of this type, carried out by several groups of workers, show that the metabolic activity of PNA and DNA nitrogen, as gaged by uptake of N^{15}, follows the same general pattern as that observed with radioactive phosphorus and carbon. For example, the uptake of heavy nitrogen by the PNA and DNA of spleen and intestine is considerably greater than the corresponding value for kidney[66-68] while in regenerating liver, the incorporation of glycine-N^{15} into the PNA and DNA is very much higher than in normal resting liver.[69,70] The ratios of uptake of labeled nitrogen into the PNA and DNA of several tissues, shown in Table V of Chapter 25, make it clear that the isotope content of the PNA is in all cases much greater than that of the DNA. In most tissues, the ratio PNA/DNA is similar to that found with other isotopes, but, in liver, the value tends to be somewhat higher than the corresponding figures for P^{32} and formate- or glycine-C^{14}. In general, where it is observed that the PNA/DNA ratio is very high, the actual isotope content of the DNA is extremely low, and even a slight error in analyses would have a marked effect on the relative values.

3. Labeled Adenine as a Precursor of PNA and DNA Purines

The ability of intact purines to serve as precursors of the nucleic acids in many organisms has been studied by several groups of workers,[64,71-73] and this subject has been extensively reviewed by Brown *et al.*,[79] by Davidson,[48,80] by Christman,[53] and by Brown.[55] From all this work, it appears

[66] P. Reichard, *Acta Chem. Scand.* **3,** 422 (1949).
[67] P. Reichard, *J. Biol. Chem.* **179,** 773 (1949).
[68] M. M. Daly, V. G. Allfrey, and A. E. Mirsky, *J. Gen. Physiol.* **36,** 173 (1952).
[69] A. Bergstrand, N. A. Eliasson, E. Hammarsten, B. Norberg, P. Reichard, and H. von Ubisch, *Cold Spring Harbor Symposia Quant. Biol.* **13,** 22 (1948).
[70] S. S. Furst and G. B. Brown, *J. Biol. Chem.* **191,** 239 (1951).
[71] A. A. Plentl and R. Schoenheimer, *J. Biol. Chem.* **153,** 203 (1944).
[72] G. B. Brown, P. M. Roll, A. A. Plentl, and L. F. Cavalieri, *J. Biol. Chem.* **172,** 469 (1948).
[73] M. E. Balis, D. H. Marrian, and G. B. Brown, *J. Am. Chem. Soc.* **73,** 3319 (1951).
[74] M. Flavin and S. Graff, *J. Biol. Chem.* **191,** 55 (1951).
[75] S. E. Kerr, K. Seraidarian, and G. B. Brown, *J. Biol. Chem.* **188,** 207 (1950).
[76] S. S. Furst, P. M. Roll, and G. B. Brown, *J. Biol. Chem.* **183,** 251 (1950).
[77] D. H. Marrian, V. L. Spicer, M. S. Balis, and G. B. Brown, *J. Biol. Chem.* **189,** 533 (1951).
[78] G. B. Brown, M. L. Peterman, and S. S. Furst, *J. Biol. Chem.* **174,** 1043 (1948).
[79] G. B. Brown, P. M. Roll, and H. Weinfeld, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 385. Johns Hopkins Press, Baltimore, 1952.
[80] J. N. Davidson, "The Biochemistry of the Nucleic Acids," 2nd. ed. Methuen, London, 1953.

that in rats and mice adenine is used as a precursor of nucleic acid adenine and, to a lesser extent, of guanine. Guanine, however, is poorly utilized by rats as a nucleic acid purine precursor and virtually no conversion of guanine to adenine takes place. In yeasts, there is considerable conversion of adenine to nucleic acid guanine, the reverse reaction not occurring to a significant extent.[79]

The metabolism of the nucleic acids in rats has been studied by Furst *et al.*[76] using adenine labeled in positions 1 and 3 with N^{15}. They observed that in whole viscera and in resting liver the PNA purines contained appreciable amounts of isotope, while incorporation into the DNA purines amounted to only about 1% of that in the PNA purines. Examination of the incorporation of labeled adenine into the PNA and DNA purines of regenerating liver showed considerable uptake of isotope by both. It was noticed that while only about 11% of the N^{15} present in the PNA at 5 days was retained at 21 days after administration of the labeled adenine, the corresponding value for DNA was 75%. This indicates a very low rate of renewal of DNA nitrogen after the period of hyperplasia. The ratios of isotope content of PNA/DNA in different tissues after administration of labeled adenine are shown in Table V of Chapter 25, and it is clear from these results that the extent of utilization of labeled adenine by DNA of resting liver is much lower than by PNA. In regenerating liver, on the other hand, or in proliferating tissues such as intestine, the PNA and DNA appear to utilize preformed adenine to about the same extent.

The uptake of adenine by the PNA and DNA of resting liver contrasts sharply with the results which have already been discussed with P^{32}-, C^{13}-, C^{14}-, and N^{15}-labeled small-molecule precursors. This discrepancy has been further investigated by Furst and Brown,[70] who have shown by the simultaneous administration of glycine-N^{15} and adenine-C^{14} that the pattern of uptake of the former by liver PNA and DNA is quite different from that obtaining with adenine. Thus while glycine is extensively utilized by both PNA and DNA, only the PNA of resting liver can be shown to contain appreciable amounts of administered adenine. That adenine can be utilized in the synthesis of DNA purines is clearly shown from experiments on regenerating liver,[70,76] on intestine,[64] and on hepatoma.[81] Furst and Brown have therefore suggested that two types of DNA, differing in metabolic activity, may be present in a single tissue. One of these DNA's may be renewed continually from small-molecule precursors while the other, which may play a proportionately greater part during rapid tissue growth, could draw upon preformed purines as precursors (see Chapter 25). Some support for the conception that two different mechanisms are available for DNA synthesis comes from the recent report[82] that, while whole body

[81] A. C. Griffin, W. E. Davis, Jr., and M. O. Tifft, *Cancer Research* **12**, 707 (1952).
[82] H. Harrington and P. S. Lavik, *Federation Proc.* **12**, 214 (1953).

irradiation causes partial inhibition of uptake of P^{32} and of labeled orotic acid and formate into the DNA of rat thymus, the incorporation of labeled adenine into the DNA adenine and guanine is not inhibited.

In view of the discrepancy between the incorporation of small-molecule precursors and preformed purines into the purines of DNA, Bendich *et al.*[83-85] have attempted to separate the tissue DNA into fractions with differing metabolic activities. Some fractionation of DNA into two products with slightly different metabolic activities has been obtained, but the difference between the fractions appears to be small in comparison with the discrepancies existing between the uptake of different precursors by DNA.

A different approach to this problem has been made by Chargaff *et al.*,[86] who have obtained several DNA fractions from calf thymus nucleohistone by the successive extraction of nucleohistone/chloroform/octanol gels with sodium chloride solutions of increasing concentration. When the relative proportions of the bases in the DNA's derived from these extracts by alcohol precipitation were determined, it was observed that the DNA obtained with increasing strengths of salt solution contained decreasing proportions of guanine and cytosine while the corresponding values for adenine and thymine increased. Similar results have been obtained independently by Brown and Watson[87] using the somewhat different technique of chromatography of calf thymus DNA on columns of histone-treated kieselguhr. By a process of stepwise elution of the DNA with increasing concentrations of sodium chloride solution, Brown and Watson obtained several separate DNA fractions which on analysis were found to contain less guanine and cytosine and more adenine and thymine as the concentration of the eluting fluid was increased. These findings suggest that DNA isolated from a tissue consists not of one or two different DNA's but of a complete family of closely related but chemically distinguishable substances which could behave metabolically in quite different ways.

4. The Metabolism of the Pyrimidine Bases

From the studies discussed in Chapter 23, it may be concluded that carbon dioxide furnishes the carbon 2 of uracil while the methyl group of

[83] A. Bendich, *Exptl. Cell Research*. Suppl. **2**, 181 (1952).
[84] A. Bendich and P. J. Russell, Jr., *Federation Proc.* **12**, 176 (1953).
[85] A. Bendich, P. J. Russell, Jr., and G. B. Brown, *J. Biol. Chem.* **203**, 305 (1953).
[86] E. Chargaff, C. F. Crampton, and R. Lipshitz, *Nature* **172**, 289 (1953).
[87] G. L. Brown and M. Watson, *Nature* **172**, 339 (1953).
[88] H. S. Loring and J. G. Pierce, *J. Biol. Chem.* **153**, 61 (1944).
[89] H. Arvidson, N. A. Eliasson, E. Hammarsten, P. Reichard, H. von Ubisch, and S. Bergstrom, *J. Biol. Chem.* **179**, 169 (1949).
[90] R. S. Hurlbert and V. R. Potter, *J. Biol. Chem.* **195**, 257 (1952).
[91] L. L. Weed and D. W. Wilson, *J. Biol. Chem.* **189**, 435 (1951).
[92] M. Edmonds, A. M. Delluva, and D. W. Wilson, *J. Biol. Chem.* **197**, 251 (1952).
[93] E. P. Anderson and S. E. G. Åqvist, *J. Biol. Chem.* **202**, 513 (1953).

thymine may be derived from the β-carbon of serine, the α-carbon of glycine, or from formate.

The subject has recently been extensively reviewed by Brown *et al.*,[79] Christman,[53] and Franke.[54] Most of the studies on pyrimidine metabolism have been made with the aid of labeled orotic acid (uracil-4-carboxylic acid). This pyrimidine has been shown to promote the growth of certain pyrimidine-deficient strains of *Neurospora*[88] and to act as a precursor of nucleic acid pyrimidines in rats,[66,89,90] in rat liver slices,[91] and in yeast.[92]

The incorporation of labeled orotic acid into rat liver and viscera nucleic acids has been studied by several workers[66,90,93] and shown to be about five times greater in a given time in PNA than DNA.

IV. Ribonucleic Acids of the Subcellular Fractions

1. Contrast between the Metabolism of Nuclear and Cytoplasmic PNA's

It has been generally accepted that the PNA of the cell is distributed amongst the various subcellular fractions (Chapter 21). While the main bulk of the PNA is found in the cytoplasmic fractions (mitochondria, microsomes, and cell sap), a small but important part is present in the cell nucleus (Chapter 18). Since these morphological fractions probably play different parts in the economy of the cell, it is clearly important to examine the relationships between the PNA's from these different sources.

In 1948, Bergstrand *et al.*[69] observed that, after administration of glycine-N^{15} to rats with normal or regenerating livers, the isotope content of the PNA from the liver cell nuclei was greatly in excess of that in the PNA of the cytoplasm. Using radioactive phosphate, Marshak and Calvet[94] followed up earlier studies on the nucleoproteins of the nucleus[95,96] and showed that the PNA of the nucleus assimilated P^{32} much more rapidly than did the PNA of the cytoplasm. Moreover, while the activity of the nuclear PNA rose rapidly after administration of the isotope and fell sharply soon afterwards, the activity of the cytoplasmic PNA increased much more slowly, reaching a maximum much later than the nuclear PNA. Similar studies have been carried out by Jeener and his co-workers,[37,39,41] Barnum *et al.*,[97,98] McIndoe and Davidson,[17] Davidson *et al.*,[99] Payne *et al.*,[10] Anderson and Åqvist,[93] Smellie *et al.*,[3] and by Tyner *et al.*,[16] who are

[94] A. Marshak and F. Calvet, *J. Cellular Comp. Physiol.* **34**, 451 (1949).
[95] A. Marshak, *J. Gen. Physiol.* **25**, 275 (1941).
[96] A. Marshak, *J. Cellular Comp. Physiol.* **32**, 381 (1948).
[97] C. P. Barnum and R. A. Huseby, *Arch. Biochem.* **29**, 7 (1950).
[98] C. P. Barnum, R. A. Huseby, and H. Vermund, *Cancer Research* **13**, 880 (1953).
[99] J. N. Davidson, W. M. McIndoe, and R. M. S. Smellie, *Biochem. J.* **49**, xxxvi (1951).

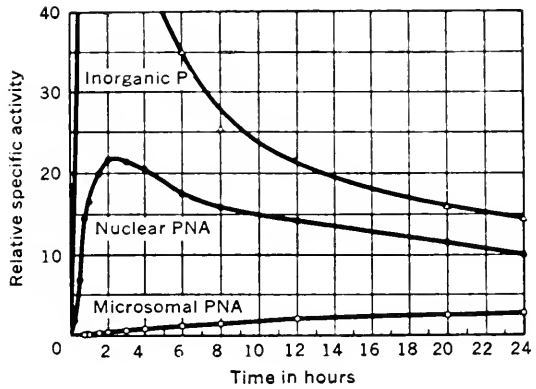

FIG. 1. Relative specific activities of the inorganic phosphate, nuclear PNA, and microsomal PNA separated from mouse liver tissue at various times after the administration of P^{32}.[97]

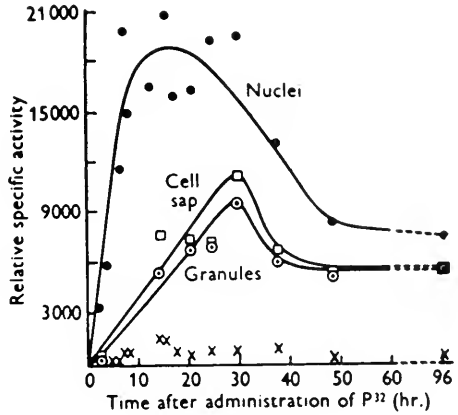

FIG. 2. Relative specific activities of the cytidylic acids of nuclear PNA, mitochondrial PNA, and microsomal PNA separated from rabbit liver at various times after the administration of P^{32}. The points for mitochondrial and microsomal PNA were coincident and the resultant curve has been termed "granules."[3]

all agreed on the very much more rapid uptake of P^{32} by nuclear than by cytoplasmic PNA at short time intervals after administration of the isotope (Figs. 1 and 2 and Table V Chapter 25).

The incorporation of radioactive phosphorus into the individual nucleotides of nuclear and cytoplasmic PNA has been studied by Marshak and Vogel,[100] McIndoe and Davidson,[17] Davidson *et al.*,[99] Smellie *et al.*,[3] and by Tyner *et al.*[16] The results show no major differences between the four

[100] A. Marshak and H. J. Vogel. *J. Cellular Comp. Physiol.* **36**, 97 (1950).

nucleotides in the nucleus or cytoplasm although adenylic acid usually exhibits the highest and guanylic acid the lowest specific activity.

Other workers have used labeled precursors of the purine and pyrimidine bases in studies on the metabolism of nuclear and cytoplasmic PNA. Potter *et al.*[101] and Hurlbert and Potter[90] have examined the uptake of orotic acid-6-C^{14} into the PNA of the nucleus and cytoplasm of rat liver from rats bearing Flexner-Jobling tumors (Fig. 3). They found that the uptake of C^{14} by the pyrimidines was much more rapid in nuclear PNA than in cytoplasmic PNA. Similar observations have been recorded by Anderson and Åqvist[93] using orotic acid-N^{15}. (see Table V Chapter 25)

Payne *et al.*[10] and Smellie *et al.*[59] using formate-C^{14} have also observed greater assimilation of the isotope into the PNA of the nucleus than into

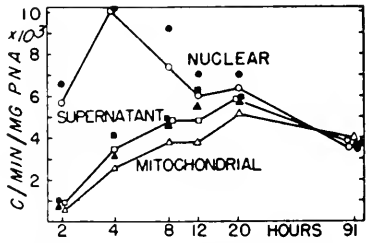

FIG. 3. The specific activities of the nuclear PNA, mitochondrial PNA, and supernatant PNA of rat liver at various times after the administration of orotic acid-6-C^{14} [90,101].

that of the cytoplasm (see Table V Chapter 25). In both nucleus and cytoplasm, adenylic acid was more heavily labeled than guanylic acid.

Glycine labeled with N^{15} [59] or C^{14} [16] has also been used to demonstrate that the uptake of isotope by nuclear PNA was greater in a given time than by cytoplasmic PNA. The difference between the PNA's from the two sources was more striking with glycine-2-C^{14} than with glycine-N^{15}.

All these observations leave no doubt as to the independent nature of the PNA of the nucleus and its considerable metabolic importance. While the proportion of the total PNA of the cell which resides in the nucleus is small, the fact that it exhibits such a relatively high metabolic activity, as evidenced by isotope experiments, means that the total turnover of nuclear PNA in a given time is not very much smaller than the total turnover of cytoplasmic PNA in the same time. The full significance of this metabolically active PNA remains something of a mystery; several suggestions have been made to account for the observed phenomenon but none of these is completely satisfactory.

[101] V. R. Potter, R. O. Recknagel, and R. B. Hurlbert, *Federation Proc.* **10**, 646 (1951).

2. The PNA's of the Cytoplasm

The distribution of PNA in the cell cytoplasm has been studied by a large number of workers and the subject has been reviewed in Chapter 21. It appears from these studies that the main bulk of the cytoplasmic PNA is located in the microsomal fraction while lesser amounts are found in the mitochondrial fraction and in the nonsedimentable material (the cell sap). The composition of the PNA's from these different cytoplasmic fractions has been shown to be the same,[102,103] and their metabolic activities have been the subject of considerable research.

Marshak and Calvet[94] have studied the uptake of P^{32} by the PNA's of the large and small particles obtained by differential centrifugation of homogenates of rabbit liver in dilute citric acid, and have observed that at time intervals up to 12 hr. the activity of the microsomal PNA exceeded that of the mitochondrial PNA. Similar results have been obtained by Jeener[37] and by Jeener and Szafarz[41] for the fractions isolated from buffered homogenates of rat liver. With mouse embryo, on the other hand, the activity of the mitochondrial PNA was found to exceed that of the microsomes. In both cases, Jeener and his co-workers have found that the activity of the nonsedimentable PNA exceeds that of either of the particulate fractions. A very thorough investigation of this topic has been made by Barnum and Huseby,[97,98] who have studied the incorporation of P^{32} into the PNA of the cytoplasmic fractions of fasted mouse liver and mammary carcinoma. These authors found no significant difference between the specific activities of the PNA's from the two particulate fractions at any time interval up to 24 hr. after administration of the isotope. At all times, however, the activity of the PNA of the cell sap was observed to be considerably higher than that of the particulate PNA. Reichard,[104] working with regenerating rat liver and glycine-N^{15}, found no difference in isotope uptake by the same base in each of the cytoplasmic fractions. Hultin *et al.*,[105] using P^{32} and glycine labeled with N^{15} in studies on the PNA of the cytoplasmic fractions of chicken liver, found that in both sets of experiments the cell sap contained more isotope than did either mitochondria or microsomes. In general, the activity of the microsomal PNA proved to be higher than that of the mitochondria, although with P^{32} the difference between the cell sap and the granules was very much greater than that between mitochondria and microsomes. Glycine-N^{15} and formate-C^{14} have been used by Smellie *et al.*[59] as PNA precursors in investigations of the relative metabolism of the PNA's from the mitochondria, microsomes, and cell

[102] G. W. Crosbie, R. M. S. Smellie, and J. N. Davidson, *Biochem. J.* **54**, 286 (1953).
[103] D. Elson and E. Chargaff, *Federation Proc.* **10**, 180 (1951).
[104] P. Reichard, *Acta Chem. Scand.* **4**, 861 (1950).
[105] T. Hultin, D. B. Slautterback, and G. Wessel. *Exptl. Cell Research.* **2**, 696 (1951).

sap. They found that incorporation of both isotopes into the PNA of the cell sap was appreciably greater than into either of the particulate fractions, the difference between which was very small. The renewal of the pyrimidine bases in the PNA from the mitochondria and residual cytoplasm of rat liver homogenates has been studied with the aid of orotic acid.[90] Here again, at various time intervals, the mitochondria exhibited lower activities than the residual cytoplasm, which was presumably composed of both microsomes and cell sap. Observations by Smellie *et al.*[3] provide results for the relative activities of the PNA's from the cytoplasmic fractions of normal, regenerating, and weanling rat liver as well as from normal, maternal, and fetal rabbit liver. In every instance the uptake of P^{32} was greatest in the PNA from the cell sap, while in normal, regenerating, or weanling rat liver the specific activities of the PNA's from the mitochondria were equal to or slightly higher than those of the PNA's from the microsomes. The same pattern was observed with normal and maternal rabbit liver, but in fetal rabbit liver the isotope content of the microsomal PNA was considerably higher than that of the mitochondrial PNA.

Tyner *et al.*,[16] in an important contribution, have examined the specific activities of the nucleotides and bases derived from the PNA of rat liver and Flexner-Jobling tumor nuclei and cytoplasmic fractions after the simultaneous administration of P^{32} and glycine-2-C^{14}. They found that at 2 hr. in both liver and tumor, as well as in the livers from tumor-bearing animals, the uptake of P^{32} and C^{14} by the PNA of the cell sap was appreciably higher than in either of the particulate fractions. The PNA of the mitochondria invariably showed lower activity than that of the microsomes at short time intervals (2 to 5 hr.), but at longer time intervals this difference became very small. Tyner *et al.*[16] also found that the specific activity of the phosphorus in the PNA nucleotides was appreciably higher than that of the carbon in the PNA bases.

Recently, Wikramanayake *et al.*[106] have followed up their previous studies[52] on the effect of different levels of energy intake on liver PNA metabolism with similar work on the PNA of the different parts of the liver cell. With animals maintained on a protein-free diet, an increase in the level of energy intake brought about an increase in the uptake of P^{32} by the PNA of the liver cell. During the first 2 hr. after administration of the isotope, this change was found to be accounted for by an increased uptake by the PNA of the nucleus. At later time intervals (4 and 18 hr.), all subcellular fractions exhibited the higher incorporation of labeled phosphorus into the PNA when the level of energy intake was raised. This effect was less marked on the PNA of the cell sap than on that of the nucleus or of the particulate fractions.

[106] T. W. Wikramanayake, F. C. Heagy, and H. N. Munro, *Biochem. et Biophys. Acta* **11**, 566 (1953).

3. The Kinetics of PNA Metabolism

Several attempts have been made to elucidate the relationship between the PNA's from the different parts of the cell by studying the specific activities of the PNA's at different times after the administration of a labeled precursor. From the work with P^{32} it is clear that the initial rate of uptake of isotope by nuclear PNA, in rabbit and mouse liver at least, is much greater than in any of the cytoplasmic fractions,[3,16,94,97,98] and that the specific activity of nuclear PNA attains a maximum which is much higher than is found with cytoplasmic PNA and is reached much earlier (Figs. 1 and 2). Similarly, the activity of nuclear PNA falls off quite rapidly, while that of the cytoplasmic PNA's takes some considerable time to fall. The specific activities of the PNA's from the different cytoplasmic fractions increase much more slowly than does that of nuclear PNA, reaching a maximum about 30 hr. after administration of the isotope. The cell sap differs slightly from the two particulate fractions in that the specific activity of its PNA is at all times higher, while the differences between the mitochondrial and microsomal PNA's are very small, particularly at the longer time intervals.[3,16,97,98]

The time-activity curves of the different PNA's with respect to the incorporation of orotic acid-6-C^{14} have been studied by Potter *et al.*,[90,101] who have again found (Fig. 3) that the specific activity of the nuclear PNA increases much more rapidly and reaches a much higher level than does that of cytoplasmic PNA. In the cytoplasm, the curve for the mitochondrial PNA was slightly lower throughout than that for the combined microsome and cell sap fractions, but both curves reached a maximum about the same time. Tyner *et al.*[16] have used glycine-2-C^{14} as a PNA precursor in experiments of this type. Once again, the incorporation into the nuclear PNA greatly exceeded that into any of the cytoplasmic PNA's. By calculating their results in absolute values, Tyner *et al.* have been able to relate directly the incorporation of P^{32} and C^{14} into the adenylic and guanylic acids of the different PNA's, and have observed that there is a greater uptake of P^{32} into the two nucleotides than of C^{14} from glycine into the adenine and guanine derived from them in normal liver.

From all this work certain conclusions may be drawn:

1. The PNA of the nucleus is much more active metabolically than the PNA from any part of the cytoplasm in liver and tumor cells.

2. In the cytoplasm, the PNA's from the mitochondria and microsomes do not differ markedly from one another in their rate of renewal.

3. The PNA of the cell sap behaves differently from either nuclear PNA or that of the two particulate fractions, although qualitatively it closely resembles the latter.

4. Experiments on the incorporation of radioactive carbon into the bases of the different PNA's have shown that the bases behave qualitatively in

a fashion similar to the phosphorus of the nucleotides. In normal liver, however, all the PNA's exhibit a greater activity with respect to phosphorus uptake than to carbon uptake.

5. It has been postulated that nuclear PNA serves as a precursor of cytoplasmic PNA (footnotes 41, 94 and 96 and Chapter 28); the results discussed here do not detract from this possibility, but they do suggest that the PNA of the nucleus is not the immediate precursor of that in the cytoplasm.

6. For most purposes it would seem that the PNA of the cell may be divided into two main metabolic classes: (a) the PNA of the nucleus which is metabolically highly active; (b) the PNA's of the cytoplasm which are qualitatively very similar but which may be slightly different quantitatively and which are much less active metabolically than the PNA of the nucleus.

The possible significance of all these observations is further discussed in Chapter 28.

V. Factors Affecting Nucleic Acid Metabolism

1. The Effect of X-Irradiation on Nucleic Acid Metabolism

a. General

It is well known that exposure of rapidly growing tissues to X-irradiation causes a marked inhibition of growth. In constrast, most adult tissues are considerably less sensitive to such exposure, and the explanation of this difference seems to lie in the relative proportion of dividing cells in the two types of tissue. Irradiation causes marked disturbances in the normal processes of cell division, and, since the nucleic acids (particularly those of the nucleus) are intimately concerned in this mechanism, it can be expected that metabolic changes in the nucleic acids will occur simultaneously with the disturbances in mitosis as a result of treatment with X-rays.

Some indications of disturbances in nucleic acid metabolism have been observed by Mitchell,[107,108] who used microspectrophotometric techniques in his studies on irradiated and non-irradiated tumor tissue. Mitchell found in the cytoplasm of the irradiated tumor cells an accumulation of ultraviolet-absorbing material which appeared to be of nucleotide nature. The effect of X-irradiation on the nucleus and cytoplasm of *Amoeba proteus* has been studied by Harriss *et al.*[109] using the technique of nuclear transfer, while Sparrow *et al.*[110] have investigated the effects of X-rays on chromosome breakage and other abnormalities in the nuclei of *Trillium erectum* and *Tradescantia paluctosa.*

[107] J. S. Mitchell, *Brit. J. Exptl. Pathol.* **23,** 285, 296, 309 (1942).
[108] J. S. Mitchell, *Brit. J. Radiol.* **16,** 339 (1943).
[109] E. B. Harriss. L. F. Lamerton, M. J. Ord, and J. F. Danielli, *Nature* **170,** 922 (1952).
[110] A. H. Sparrow, M. J. Moses, and R. J. DuBow, *Exptl. Cell Research* Suppl. 2, 245 (1952).

b. The effect of X-irradiation on the Uptake of Isotopes by the Nucleic Acids of the Irradiated Tissue

Hevesy[111,112] has studied the uptake of P^{32} by the DNA of Jensen rat sarcoma after doses of X-rays of about 1000 r. and has shown that isotope administered immediately after irradiation is taken up much more slowly in the irradiated tumor than in non-irradiated controls. A similar degree of inhibition (60 to 70%) of DNA renewal was observed in normal rat liver, spleen, and intestinal mucosa after irradiation. When the administration of the labeled phosphorus was delayed until several days after exposure to the X-rays, the difference between the specific activity of the DNA's in the control and irradiated tumors was very much smaller than when the isotope was given immediately after irradiation; indeed it was observed in some experiments that 75% of the blocking effect of the X-rays disappeared within 2 hr. of exposure to the radiation. The rapid diminution in effectiveness of X-rays as a means of blocking DNA renewal may well explain the greater sensitivity of growing tissues to irradiation, since, in such tissues, the frequency of mitosis and the consequent synthesis of DNA is much greater than in fully grown tissues where mitosis is relatively rare. It follows that, if the effect of irradiation is only of short duration, few cells would be damaged in adult or resting tissues, whereas many more would be affected in a proliferating tissue.

In weanling rat liver, the degree of blocking of P^{32} uptake into the DNA by X-irradiation immediately prior to administration of the isotope, is similar to that found in adult animals.[111] When the animals were irradiated continuously from the time of injection of isotopic phosphorus until sacrifice, the ratio of activity in the control and treated animals was found to be as high as 11 to 1.

Similar results on the inhibition of P^{32} incorporation into the DNA of rat Jensen sarcoma have been recorded by Holmes,[113-115] who has also observed a lesser degree of inhibition of phosphorus uptake by the PNA of the tumor. Inhibition of DNA synthesis in bean root has been observed by Howard and Pelc.[5]

Hevesy,[111,112] Holmes,[30,113,114] and Kelly and Jones[116] have noted an indirect effect in an animal with an irradiated and a control tumor whereby the uptake of P^{32} by the control tumor was substantially reduced, although to a lesser extent than in the irradiated tumor. In an endeavor to determine

[111] G. C. Hevesy, *Revs. Mod. Phys.* **17,** 102 (1945).
[112] G. C. Hevesy, *J. Chem. Soc.* **1951,** 1618.
[113] B. E. Holmes, *Brit. J. Radiol.* **20,** 450 (1947).
[114] B. E. Holmes, *Brit. J. Radiol.* **25,** 273 (1952).
[115] B. E. Holmes, *Ciba Conf. on Isotopes in Biochem., London* p. 114 (1951).
[116] L. S. Kelly and H. B. Jones, *Proc. Soc. Exptl. Biol. Med.* **74,** 493, (1950).

the nature of this effect, Ahlstrom *et al.*[117] have transfused the blood of an irradiated rabbit into an untreated animal. The results of this experiment were not conclusive, but it was found that in the kidney of the recipient animal the uptake of P^{32} by the DNA was lowered by the transfusion, as might be expected if some humoral agent were present.

The possibility that the effect of irradiation on DNA metabolism is concerned only with the phosphorylation processes has been ruled out by the work of Hevesy,[118] who found that the incorporation of acetate-1-C^{14} into the DNA purines was reduced by irradiation to about the same extent as the reduction in P^{32} incorporation. Abrams[119] in similar experiments using glycine-1-C^{14} has found that the incorporation of radioactive carbon into the PNA and DNA purines of rabbit bone marrow and intestine, and of rat intestine, was greatly reduced by X-irradiation. Harrington and Lavik[82] have observed that while whole body irradiation partially inhibits the incorporation of small-molecule precursors into the DNA of rat thymus, the uptake of labeled adenine is not affected.

The effects of X-rays on the metabolism of the PNA of the nucleus and cytoplasm as well as on the DNA of rat liver cells has been studied by Payne *et al.*,[120] who have shown in two strains of rats and also in mice that the incorporation of P^{32} into the cytoplasmic PNA was increased after irradiation, while the uptake of isotope by nuclear PNA and DNA was greatly diminished.

Recently Vermund *et al.*[121] have studied the effects of X-irradiation on the incorporation of P^{32} into the nucleic acids of transplanted mouse mammary carcinomata. They found that irradiation caused a marked fall in the uptake of the isotope by the DNA of the irradiated tumor. A less-marked inhibition of DNA metabolism in a shielded tumor in the same animal was also observed. No statistical evidence was found however to show that the irradiation has any action on the metabolism of PNA of the tumor nuclei or cytoplasmic fractions.

2. The Effect of Antibiotics on Nucleic Acid Metabolism

a. Penicillin

In 1947, Krampitz and Werkman[122] showed that in high concentrations penicillin brought about inhibition of the oxidation of the pentose con-

[117] L. Ahlstrom, H. von Euler, G. C. Hevsey, and K. Zerahn, *Arkiv Kemi, Mineral. Geol.* **23A**, 1 (1946).

[118] G. C. Hevesy, *Nature* **163**, 869 (1949).

[119] R. Abrams, *Arch. Biochem.* **30**, 90 (1951).

[120] A. H. Payne, L. S. Kelly, and C. Entenman, *Proc. Soc. Exptl. Biol. Med.* **81**, 698 (1952).

[121] H. Vermund, C. P. Barnum, R. A Huseby, and K W. Stenstrom, *Cancer Research* **13**, 633 (1953).

[122] L. O. Krampitz and C. H. Werkman, *Arch. Biochem.* **12**, 57 (1947).

stituent of PNA by lyophilized preparations of *Staphylococcus aureus*. About the same time, Gros and Macheboeuf[123-125] found that penicillin exhibited an inhibitory effect on the catabolism of adenylic, guanylic, and uridylic acids by *Clostridium sporogenes*. Soon after these findings, Mitchell *et al.*[126,127] observed that, immediately after the addition of penicillin to growing cultures of *S. aureus*, a progressive disturbance occurred in the relationship between extractable nucleotides and the nucleic acids of the organisms, due apparently to a very considerable increase in the amount of nucleotides extractable from the cells. This accumulation appeared to be the result of a decreased rate of incorporation into the nucleic acid molecules rather than to an increase in the rate of synthesis of nucleotides. Park and Johnson,[128] in studies on the nucleotide fraction of *S. aureus* growing in the presence and absence of penicillin, noticed an accumulation of labile organic phosphate with an absorption maximum at 262 mμ. Subsequent work by Park[129-133] resulted in the isolation of three uridine pyrophosphate derivatives from acid extracts of the penicillin-treated cells. The first of these compounds was shown to be uridine pyrophosphate attached to an *N*-acetylamino sugar, while the second contained the uridine pyrophosphate *N*-acetylamino sugar combined with L-alanine. The third derivative, which may not be a single substance, appeared to consist of a peptide attached to the uridine pyrophosphate *N*-acetylamino sugar as before. This peptide is composed of one L-lysine, one D-glutamic acid, and three alanine residues. Park[133] suggests that these compounds may occur naturally in normal *S. aureus* cells but that they accumulate in penicillin-treated cells because of an inability to metabolize them further. In an attempt to isolate these substances from normal cells, Park obtained a very small amount of three substances which corresponded to the three isolated from the penicillin-treated cells, but there was insufficient material to prove that the two groups of material were identical. However, if, as seems likely, these three substances are present even in minute amounts in normal bacterial cells, it is reasonable to suppose that penicillin in some way affects the system which normally metabolizes them. From the quan-

[123] F. Gros and M. Macheboeuf, *Ann. inst. Pasteur.* **74,** 308 (1948).
[124] F. Gros and M. Macheboeuf, *Bull. acad. méd. (Paris)* **5,** 80 (1948).
[125] F. Gros and M. Macheboeuf, *Compt. rend.* **224,** 858 (1949).
[126] P. Mitchell, *Nature* **164,** 259 (1949).
[127] P. Mitchell and J. Moyle, *J. Gen. Microbiol.* **5,** 421 (1951).
[128] J. T. Park and M. J. Johnson. *J. Biol. Chem.* **179,** 585 (1949).
[129] J. T. Park, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 1, p. 93. Johns Hopkins Press, Baltimore, 1951.
[130] J. T. Park, *J. Biol. Chem.* **194,** 877 (1952).
[131] J. T. Park, *J. Biol. Chem.* **194,** 885 (1952).
[132] J. T. Park, *J. Biol. Chem.* **194,** 897 (1952).
[133] J. T. Park, *2nd Intern. Congr. Biochem., Paris* p. 31 (1952).

titative point of view, it is interesting to observe that the amount of these derivatives of uracil present in the extracts of penicillin-treated cells must represent a considerable proportion of the uracil which would be required for the synthesis of PNA, and that on this basis one might expect to find considerable inhibition of PNA synthesis.

Gros and Macheboeuf[134] have recently followed up their previous observations on the inhibition of the catabolism of certain nucleotides by the treatment of *S. aureus* with penicillin, by an investigation of the ability of nongrowing preparations of the organism to catabolize PNA, and various derivatives of PNA, in the presence and absence of penicillin. They found that there was appreciable inhibition of the oxidation of PNA, guanylic acid, guanosine, and uridylic acid by *S. aureus* in the presence of high concentrations of penicillin. In the same conditions, however, the metabolism of the other nucleotides, nucleosides, or of ribose and ribose-5-phosphate was unaffected. It was therefore concluded that penicillin affects the nucleosidase activity of the organism although very considerable differences were found with different strains of the organism.

These authors[134] have also studied the effect of small doses of the antibiotic on growing cultures of *S. aureus*. In this work it was found that cells which had been treated with penicillin during the growth period and were then separated from the medium and washed, showed greatly reduced ability to oxidize guanosine or guanylic acid as compared with the normal.

Gale and Folkes[135-137] have also investigated the mode of action of penicillin on the synthesis of nucleic acids by *S. aureus*. They found that this organism could synthesize nucleic acids when the medium contained an amino acid mixture. If this amino acid mixture were supplemented by a mixture of purines and pyrimidines, the synthesis of nucleic acids was greatly stimulated. High concentrations of penicillin (50 to 10,000 units per ml.) caused some inhibition of the additional nucleic acid synthesis, stimulated by the purine–pyrimidine mixture, without any effect on the basic synthesis of nucleic acids.

b. Aureomycin, Chloramphenicol, Neomycin, and Terramycin

These four antibiotics fall into one class; at very high concentrations they inhibit nucleic acid synthesis in *S. aureus*, but, at concentrations corresponding to those producing complete inhibition of growth, synthesis of nucleic acids is actually stimulated.[135-137]

[134] F. Gros and M. Macheboeuf, *2nd Intern. Congr. Biochem., Paris* p. 101 (1952).
[135] E. F. Gale and J. P. Folkes, *Biochem. J.* **53,** 493 (1953).
[136] E. F. Gale, *2nd Intern. Congr. Biochem., Paris* p. 5 (1952).
[137] E. F. Gale, *Advances in Protein Chem.* **8,** 285 (1953).

c. Bacitracin, Polymyxin, and Streptomycin

These three substances are apparently without much effect on the synthesis of nucleic acids by *S. aureus* although bacitracin in enormous concentrations (2 to 10 mg./ml.) does have a marked inhibitory action.[135-137]

VI. The Metabolism of Virus Nucleic Acids

1. The Origin of Virus Nucleic Acids

It is well known that virus particles are rich in nucleic acid, and that in an infected organism very considerable multiplication of virus particles takes place. It is obvious therefore that a large amount of nucleic acid must be synthesized in the course of this process. A useful account of several aspects of virus multiplication is given in the report of a recent Symposium of the Society for General Microbiology[138] and in a review by Putnam.[139] A particularly good system for the study of the origin and fate of virus nucleic acids has proved to be the *Eschcrichia coli*-bacteriophage systems which have been extensively employed to elucidate these reactions.

Many aspects of the infection of *E. coli* with bacteriophage T_2 have been discussed by Heden,[140] and an excellent summary of the great volume of isotope studies on bacterial viruses has recently been produced by Evans.[141]

a. The Source of Bacteriophage Phosphorus

Experiments have been carried out by Kozloff and Putnam[142,143] and Evans[144] with the *E. coli* T_6r^+ system in which either *E. coli* cells labeled with P^{32} were incubated in an isotope-free medium with preprations of the T_6 bacteriophage, or unlabeled bacterial cells were incubated in a P^{32}-labeled medium with the phage particles. The virus particles produced in these systems were then isolated and their isotope content measured.

From experiments of this kind it was found that in synthetic medium and in nutrient broth about 75% of the virus DNA phosphorus was derived from the phosphorus of the medium, while 17 to 31% originated in the DNA phosphorus of the host cells. These observations are in accord with those of Cohen[145] on the uptake of P^{32} by bacteriophage T_2 and T_4 and with the

[138] P. Fildes and W. E. van Heyningen (eds.), *2nd Symposium Soc. Gen. Microbiol. Cambridge* (1953).
[139] F. W. Putnam, *Advances in Protein Chem.* **8,** 175 (1953).
[140] C. G. Heden, *Acta Pathol. Microbiol. Scand.* Suppl. 88 (1951).
[141] E. A. Evans, "Biochemical Studies of Bacterial Viruses." Univ. of Chicago Press, Chicago, 1952.
[142] L. M. Kozloff and F. W. Putnam, *J. Biol. Chem.* **182,** 229 (1950).
[143] F. W. Putnam and L. M. Kozloff, *Science* **108,** 386 (1948).
[144] E. A. Evans, *Bacteriol. Revs.* **14,** 210 (1950).
[145] S. S. Cohen, *J. Biol. Chem.* **174,** 295 (1948).

more general studies of Cohen[146,147] on the synthesis of nucleic acids by bacteriophate T_2, T_4, and T_6.

b. Bacteriophage Nucleic Acids Studied with N^{15} and C^{14}

Kozloff *et al.*[148] and Siddiqi *et al.*,[149] in studies on the *E. coli* T_6 bacteriophage system with the aid of N^{15}-labeled host cells and medium, have made observations closely confirming those made with P^{32}. Thus when the T_6r^+ phage was grown on unlabeled bacteria in an N^{15}-labeled medium, about 66% of the virus DNA formed was found to be derived from the medium, while in the complementary experiment in which the bacteria were labeled, from 16 to 40% of the virus nucleic acid nitrogen originated in the nitrogen of the infected cells. Similar amounts of bacterial nitrogen were found in each of the bases derived from the virus nucleic acid. The extent of transfer of bacterial nitrogen to virus nitrogen was decreased when the nitrogen content of the medium was raised by the addition of ammonium chloride or purine bases, but, even in the presence of a large excess of medium nitrogen, considerable transfer of bacterial nitrogen to the viral progeny occurred.

In experiments in which the bacteria were labeled with N^{15} and P^{32}, it was noted that there was a relatively greater transfer of bacterial nitrogen than of bacterial phosphorus to the virus DNA, suggesting that, while the transfer may involve the bases of the bacterial DNA, it is probably not purely a transfer of nucleotides or polynucleotides.

Koch *et al.*[150] have investigated the transfer of host purines to the phage progeny using bacteria in which the purines alone were labeled with C^{14}. It was found that from 14 to 24% of the phage adenine and 20 to 35% of the phage guanine was derived from the bacterial purines and that only the purines of the phage were significantly labeled. In other experiments where the bacterial purines were labeled with C^{14} and the remaining nitrogen of the bacteria with N^{15}, it was apparent that only the purines of the bacteria were converted to phage purines. Similar utilization of host pyrimidines in the formation of phage pyrimidines has been noted by Weed and Cohen.[151]

Recently Putnam and his co-workers[152,153] have extended their studies of

[146] S. S. Cohen, *J. Biol. Chem.* **174,** 281 (1948).
[147] S. S. Cohen, *Bacteriol. Revs.* **15,** 131 (1951).
[148] L. M. Kozloff, K. Knowlton, F. W. Putnam, and E. A. Evans, *J. Biol. Chem.* **188,** 101 (1951).
[149] M. S. H. Siddiqi, L. M. Kozloff, F. W. Putnam, and E. A. Evans, *J. Biol. Chem.* **199,** 165 (1952).
[150] A. L. Koch, F. W. Putnam, and E. A. Evans, *J. Biol. Chem.* **197,** 113 (1952).
[151] L. L. Weed and S. S. Cohen. *J. Biol. Chem.* **192,** 693 (1951).
[152] F. W. Putnam, D. Miller, L. Palm, and E. A. Evans, *J. Biol. Chem.* **199,** 177 (1952).
[153] F. W. Putnam, *Exptl. Cell Research.* Suppl. 2, 345 (1952).

the T_6 bacteriophage to the smaller T_7 phage, using methods essentially the same as those used with the larger virus. As with the T_6 phage, it was found that there is considerable transfer of host nucleic acid nitrogen and phosphorus to the viral progeny. The T_7 phage differs from the T_6 in that the latter synthesizes a high proportion of its DNA from products in the medium, while the former, being smaller, has most of its requirement for DNA met by the DNA of the infected cells, and there is little utilization of components of the medium for DNA synthesis.

2. The Fate of the Infecting Virus Particle

Kozloff, in a recent review,[154] and Putnam and Kozloff[155] have examined the fate of the phosphorus of the infecting T_6r^+ phage particle by preparing P^{32}-labeled samples of the virus and using them to infect unlabeled cells in an isotope-free medium. It was shown that 50 to 60% of the isotope appeared in the medium as low-molecular-weight compounds, and the evidence suggests that the infecting particle is disintegrated on infecting the bacterial cell. Of the remaining phosphorus, 10 to 15% was associated with the bacterial debris (perhaps due to absorption of the newly formed phage onto bacterial debris), while 20 to 40% appeared in the viral progeny. The liberation of acid-soluble phosphorus from infecting T_2r^+ phage has been observed by Lesley *et al.*[156-158] who have studied the liberation of P^{32} from labeled virus particles at different times after infection with different proportions of virus per bacterial cell.

These mechanisms have been further investigated by Kozloff[159,160] using T_6r^+ phage labeled with N^{15} or with N^{15} and P^{32}. Again, there was found to be a transfer of nitrogen from the parent phage to progeny amounting to about 20% of the original isotope content of the infecting particle. Sixty to 80% of the remaining nitrogen was broken down to small molecules and appeared in the medium. In another experiment labeled bacteria were infected with phage and the phage progeny (labeled only in the DNA derived from the bacteria) were isolated. These labeled phage were then used to infect more unlabeled bacteria, when it was found that the proportion of parent P^{32} appearing in the progeny was the same as in completely labeled phage, indicating that the DNA of the phage which is derived from the host, and that synthesized from the medium, participate to the same

[154] L. M. Kozloff, *Exptl. Cell Research.* Suppl. 2, 367 (1952).
[155] F. W. Putnam and L. M. Kozloff, *J. Biol. Chem.* **182,** 243 (1950).
[156] S. M. Lesley, R. C. French, A. F. Graham, and C. E. van Rooyen, *Can. J. Med. Sci.* **29,** 128 (1951).
[157] S. M. Lesley, R. C. French, and A. F. Graham. *Arch. Biochem.* **28,** 149 (1950).
[158] R. C. French, A. F. Graham, S. M. Lesley, and C. E. van Rooyen, *J. Bacteriol.* **64,** 597 (1952).
[159] L. M. Kozloff, *J. Biol. Chem.* **194,** 83 (1952).
[160] L. M. Kozloff, *J. Biol. Chem.* **194,** 95 (1952).

extent in the reactions in the infected cell. From these observations and results obtained with phage inactivated by exposure to ultraviolet and X-irradiation and also from systems infected with both T_6 and T_7 phage, it appears that much of the parent material appearing in the progeny is derived from breakdown products of the parent phage in the medium, and that there is little transfer of genetic units from parent phage to progeny.

Similar experiments carried out with T_2r^+, T_4r, and T_3 phages labeled with P^{32} and C^{14} by Watson and Maaloe[161] indicated that about 45% of the parent isotope was incorporated in the progeny, 5 to 10% was associated with bacterial debris, and the remainder was in the nonsedimentable fraction. It was found that the transmission of P^{32} from the parent phage to progeny occurred mainly in the early formed phage and that the subsequent particles received little isotope from the parent.

Hershey and Chase,[162] using T_2 phage labeled with P^{32} and S^{35}, observed that on infection most of the phage DNA enters the infected cell while a residue, containing most of the phage sulfur-containing protein, remains attached to the surface of the infected cell.

In a later paper Hershey[163] has followed the synthesis of T_2 bacteriophage in P^{32}-labeled systems. He concludes that on infection a pool of precursor DNA is built up which is subsequently incorporated into the phage particles. The kinetics of transport of phosphorus from the culture medium, bacterial DNA, and infecting phage DNA to the viral progeny have been studied, and it has been noted that the phosphorus of the mature phage does not exchange with that in the precursor. See also p. 460.

The *S. muscae* bacteriophage system has been investigated by Price,[164] who has found that in certain circumstances absorption of the virus particles kills the bacterial cells, no virus material being synthesized. In certain strains of *S. muscae*, phage particles are released before lysis occurs, while in other strains cellular lysis and release of virus particles occur simultaneously.

VII. The Catabolism of the Nucleic Acids

1. General

Ingested nucleic acids are broken down in the intestine under the influence of the enzymes already discussed in Chapter 15 and recently reviewed by Christman,[53] Davidson,[80,165] and Laskowski,[166] yielding free bases, phosphoric acid, and the free sugar.

[161] J. D. Watson and O. Maaloe, *Biochem. et Biophys. Acta* **10**, 432 (1953).
[162] A. D. Hershey and M. Chase, *J. Gen. Physiol.* **36**, 39 (1952).
[163] A. D. Hershey, *J. Gen. Physiol.* **37**, 1 (1953).
[164] W. H. Price, *J. Gen. Physiol.* **35**, 409 (1952).
[165] J. N. Davidson, *Brit. Med. Bull.* **9**, 154 (1953).
[166] M. Laskowski, *in* "The Enzymes" (Sumner and Myrbäck, eds.), Vol. 1, Part 2, p. 956. Academic Press, New York, 1951.

2. The Degradation of the Purine Bases

a. The Formation and Breakdown of Uric Acid

The initial step in the breakdown of the purine bases is one of deamination, the nature of the deaminases responsible having been discussed in Chapter 15, and also by Christman[53] and Laskowski.[166]

Most tissues contain relatively little adenase (adenine deaminase) although adenosine deaminase is more widely distributed: in contrast, guanase (guanine deaminase) occurs more generally in animal tissues. Adenosine may be deaminated to inosine before the sugar moiety is split off, while with guanosine, the base sugar linkage may be broken before deamination of the base takes place. In any event, the net result of these reactions is the production of hypoxanthine and xanthine from adenosine and guanosine, respectively (Fig. 4). The hypoxanthine and xanthine so formed are oxidized by xanthine oxidase to uric acid, the same enzyme apparently being responsible for the oxidation of hypoxanthine to xanthine and of xanthine to uric acid (Fig. 4). Some doubt exists as to whether xanthine oxidase is the only system concerned in the formation of uric acid from xanthine and hypoxanthine, since it has been found that in rats maintained on a protein-free diet there is a marked decrease in the xanthine oxidase activity of the liver and to a lesser extent of the kidneys without there being any major fall in the ability of the animal to oxidize xanthine.[167,168]

In most mammals, uric acid is not the final end-product of purine catabolism, for it is further oxidized to the much more soluble allantoin by the enzyme uricase. The exceptions to this general rule are man, other primates, and the Dalmatian dog (for recent reviews, see Christman[53] and Franke[54]). The anomaly in the metabolism of uric acid by the Dalmatian coach-hound has been the subject of much research, from which it appears that the uricase content of the livers of Dalmatian and other dogs is similar[169] and that the excretion of uric acid by the former is not due to an inability of the animal to convert uric acid to allantoin, but rather to an abnormality of kidney function. Friedman and Byers[170] consider that the kidney of the Dalmatian dog can clear uric acid more rapidly than can that of other dogs but that there is little tubular reabsorption. Wolfson *et al.*,[171] on the other hand, believe that there is active tubular excretion of uric acid in the kidney of the Dalmatian dog as distinct from other breeds.

The oxidation of uric acid to allantoin (Fig. 4) has been extensively

[167] J. N. Williams, P. Fiegelson, and C. A. Elvehjem, *J. Biol. Chem.* **185,** 887 (1950).
[168] A. D. Bass, J. Tepperman, D. A. Richert, and W. W. Westerfeld, *Proc. Soc. Exptl. Biol. Med.* **73,** 687 (1950).
[169] F. W. Klemperer, H. C. Trimble, and A. B. Hastings, *J. Biol. Chem.* **125,** 445 (1938).
[170] M. Friedman and S. O. Byres, *J. Biol. Chem.* **175,** 727 (1948).
[171] W. O. Wolfson, C. Cohn, and C. Shore, *J. Exptl. Med.* **92,** 121 (1950).

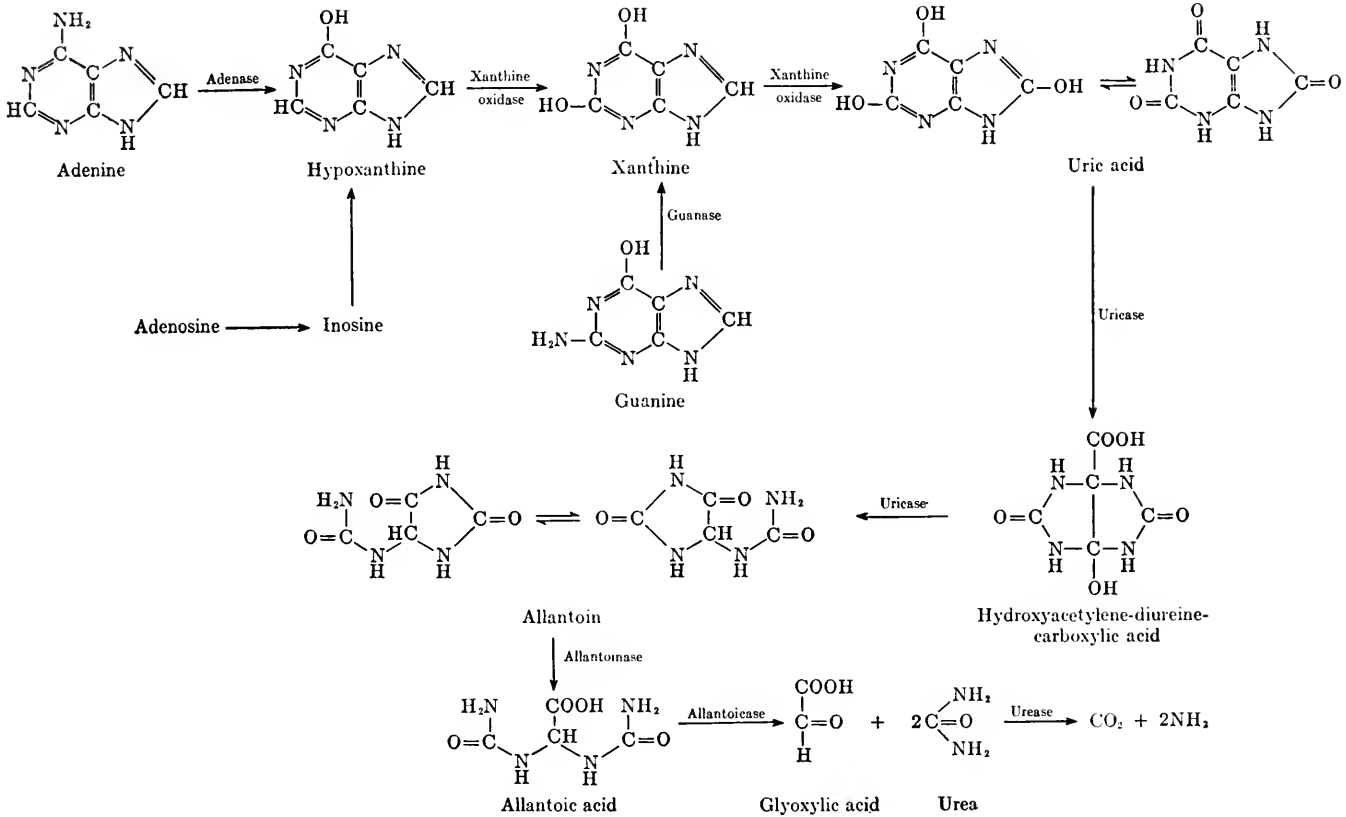

FIG. 4. The catabolism of the purines.

Uric acid	Man and other primates, birds, terrestrial reptiles Cyclostomes Insects (except Diptera)
(*Uricase*) ↓ Allantoin	Mammals (except man and other primates) Diptera Gastropods
(*Allantoinase*) ↓ Allantoic acid	One group of Teleosts (Salmonidae, Pleuronectidae, Anguillidae)
(*Allantoicase*) ↓ Urea	Selachii, Dipnoi, Crossopterygii One group of Teleosts (Cyprinidae, Esocidae, and Scombridae) Amphibia Fresh-water lamellibranchs
(*Urease*) ↓ Ammonia	Sipunculids Marine lamellibranchs Crustacea

FIG. 5. Comparative biochemistry of the breakdown of uric acid (Florkin[175]).

studied, and it would appear from the earlier studies recently summarized by Christman,[53] Franke,[54] and Klemperer[172] and from the more recent work of Brown and his co-workers[72,173,174] that a symmetrical product such as hydroxyacetylene-diureine-carboxylic acid may be produced as an intermediate in this reaction (Fig. 4). Brown's studies have shown that after the administration of adenine or uric acid labeled with N^{15} in the 1- and 3-positions, the isotope content of the excreted allantoin is evenly distributed between the imidazole ring and the urea side chain. This would be possible only if a rearrangement of the molecule between uric acid and allantoin had taken place.

Allantoin is the main end-product of purine metabolism in most mammals. In some fish, the allantoin is further broken down by allantoinase to allantoic acid (Figs. 4 and 5), while other fish and amphibia split the allantoic acid to urea and glyoxylic acid by the action of allantoicase.[175-177]

[172] F. W. Klemperer, *J. Biol. Chem.* **160,** 111 (1945).
[173] G. B. Brown, P. M. Roll, and L. F. Cavalieri, *J. Biol. Chem.* **171,** 835 (1947).
[174] G. B. Brown, *Cold Spring Harbor Symposia Quant. Biol.* **13,** 43 (1948).
[175] M. Florkin, "Biochemical Evolution" (edited, trans., and augmented by S. Morgulis). Academic Press, New York, 1949.
[176] E. Baldwin, "An Introduction to Comparative Biochemistry." Cambridge Univ. Press, England, 1949.

Still lower forms of animal life degrade the urea to ammonia and carbon dioxide by means of the enzyme urease, thus reducing the nucleic acid purine to the simplest of units. A large number of enzymes is concerned in this process of degradation and their distribution has been summarized by Florkin,[175] Baldwin,[176] and Laskowski,[177] and also in Chapter 15 of this volume.

In birds and reptiles, the chief end-product of all nitrogen metabolism is uric acid. This probably represents an attempt to spare the water which would be required in the excretion of other more soluble nitrogenous end-products. The synthesis of uric acid and tissue purines in pigeons has been studied by Barnes and Schoenheimer[178] using ammonium citrate labeled with N^{15}. They found considerable incorporation of the isotope into the tissue purines as well as into the excreted uric acid, and conclude from their calculations that the synthesis of uric acid from ammonia in the pigeon (the major pathway for the excretion of nitrogen) passes through the purines of at least some of the tissue nucleic acids. The catabolism of purines by several microorganisms has been reviewed by Brown.[55]

b. The Metabolism of Uric Acid

The metabolism of uric acid has recently been reviewed by Bishop and Talbott.[179] In man and in certain higher primates in contrast to most other mammals, uric acid appears to be the main end-product of purine metabolism; only small quantities of allantoin, possibly of dietary origin, appear in the urine. This anomaly in purine metabolism is due to the absence of uricase from the tissues of these species. Geren *et al.*[180] have studied the fate of N^{15}-labeled uric acid administered orally and intravenously to normal men and have found that orally administered uric acid is largely degraded to urea, whereas the bulk of the intravenous uric acid is excreted unchanged. Other workers[181] have found that, after intravenous injection of N^{15}-labeled uric acid, only 68 to 77% of the administered isotope was recovered in the uric acid of the urine. Alternative metabolic pathways would therefore appear to exist. Recently, Wyngaarden and Stetten[182] have carried out similar studies from which they conclude that about 18% of the administered uric acid is degraded to urea and ammonia and some 6% appears in the feces. Repetition of this experiment in a subject in whom

[177] M. Laskowski, *in* "The Enzymes," (Sumner and Myrbäck, eds.), Vol. 1, Part 2, p. 946. Academic Press, New York, 1951.

[178] F. W. Barnes, Jr., and R. Schoenheimer, *J. Biol. Chem.* **151,** 123 (1943).

[179] C. Bishop and J. H. Talbott, *Pharmacol. Revs.* **5,** 231 (1953).

[180] W. D. Geren, A. Bendich, O. Bodansky, and G. B. Brown, *J. Biol. Chem.* **183,** 21 (1950).

[181] J. Buzard, C. Bishop, and J. H. Talbott, *J. Biol. Chem.* **196,** 179 (1952).

[182] J. B. Wyngaarden and DeW. Stetten, Jr., *J. Biol. Chem.* **203,** 9 (1953).

intestinal bacteriostasis was maintained by means of oral sulfonamides, showed that the intestinal flora are not responsible for the observed partial breakdown of uric acid.

It has long been recognized that there is a pathological accumulation of uric acid or urates in human gout, and this observation has stimulated further research into the metabolism of uric acid in man. Thus the amount of the uric acid pool in normal and gouty individuals has been determined by several groups of workers,[180,181,183,184] who have found that, in the normal man, the size of the pool of miscible uric acid is about 1000 mg. of which between 60 and 80% is renewed each day. In gouty subjects, on the other hand, the size of this miscible pool is increased, generally to upwards of 2000 mg. while the turnover is of the order of 50% per day. The effects of treatment with colchicine, ACTH, and cortisone on the size and turnover of the uric acid pool have been discussed by Bishop *et al.*[183] Benedict *et al.*,[185] in an attempt to study the metabolic defect which leads to an increased uric acid pool, have observed that after administration of glycine-N^{15} there is initially a greater rise, and subsequently a more rapid fall, in the isotope content of the uric acid from gouty, as distinct from normal, patients. These authors interpret this to mean that the uric acid in patients with gout is formed more rapidly and possibly more directly from glycine than normal, and that the increase in size of the uric acid pool is due to an overproduction of uric acid rather than to a failure in the mechanism of uric acid excretion.

3. The Catabolism of the Pyrimidines

Very much less is known concerning the catabolism of the pyrimidines than of the purines. The feeding experiments of Cerecedo and his co-workers[186-192] have indicated that, except for cytosine which is not readily absorbed, the pyrimidines are rapidly oxidized and excreted as urea and oxalic acid. Plentl and Schoenheimer,[71] using uracil and thymine labeled with N^{15}, found that the administered pyrimidines are broken down and excreted as urea and ammonia, the urinary allantoin having negligible isotope con-

[183] C. Bishop, W. Garner, and J. H. Talbott, *J. Clin. Invest.* **30,** 879 (1951).
[184] J. D. Benedict, P. H. Forsham, and DeW. Stetten, Jr., *J. Biol. Chem.* **181,** 183 (1949).
[185] J. D. Benedict, M. Roche, T. F. Yu, E. J. Bien, A. Gutman, and D. Stetten, *Metabolism.* **1,** 3 (1952).
[186] L. R. Cerecedo, *J. Biol. Chem.* **75,** 661 (1927).
[187] L. R. Cerecedo, *J. Biol. Chem.* **88,** 695 (1930).
[188] L. R. Cerecedo, *J. Biol. Chem.* **93,** 283 (1931).
[189] L. R. Cerecedo and O. H. Emerson, *J. Biol. Chem.* **87,** 453 (1930).
[190] O. H. Emerson and L. R. Cerecedo, *Proc. Soc. Exptl. Biol. Med.* **27,** 203 (1929).
[191] J. A. Stekol and L. R. Cerecedo, *J. Biol. Chem.* **93,** 275 (1931).
[192] J. A. Stekol and L. R. Cerecedo, *J. Biol. Chem.* **100,** 653 (1933).

tent. Bendich *et al.*[193] have observed that nearly 90% of administered cytosine is eliminated in the urine of rats within 3 days, much of it in the form of urea and ammonia.

The pathway of pyrimidine breakdown in dogs proposed by Cerecedo[187,188] envisages the conversion of uracil to isobarbituric acid, to isodialuric acid, to formyloxaluric acid, to formic acid plus oxaluric acid, and finally to oxalic acid plus urea. Thymine, on the other hand, is converted to thymine glycol and finally yields carbon dioxide and urea. Recent studies on the catabolism of pyrimidines by bacteria have been carried out by Hayaishi and Kornberg,[194,195] Wang and Lampen,[196-198] and by Lara.[199,200] The results obtained by these workers with bacteria suggest that thymine glycol is not an intermediate in the breakdown process, but that thymine is converted to 5-methylbarbituric acid while uracil is oxidized to barbituric acid (Fig. 6). The oxidation of the two substrates has been studied using enzyme preparations from cell-free extracts, and it has been shown that the same enzyme (uracil-thymine oxidase) is responsible for both reactions.[195,199] Analysis of the products of reaction of this enzyme on thymine and uracil have been carried out by Hayaishi and Kornberg,[195] who have found that the substances produced exhibit the ultraviolet spectra and ion-exchange properties of 5-methylbarbituric acid and barbituric acid, respectively. Lara[199] has found that, in some instances, thymine may be demethylated to uracil, but that this is not the normal pathway for the oxidation of thymine. This pathway also appears to be that used in the oxidation of the two aminopyrimidines, cytosine and 5-methylcytosine, which are first deaminated by an adaptive enzyme to uracil and thymine, respectively.[195] These oxidation pathways are illustrated in the first part of Fig. 6.

The fate of the barbituric acid formed from uracil and cytosine has also been studied using preparations of the enzyme (barbiturase) from cell-free extracts of bacteria.[195,199] Barbituric acid is broken down to malonic acid and urea, which is further hydrolyzed under the influence of urease to carbon dioxide and ammonia (Fig. 5). The further degradation of 5-methylbarbituric acid remains unknown; it is readily destroyed by the intact cells under ærobic conditions, but cell-free extracts exhibit no activity towards this substrate. It seems not unlikely, however, that the 5-methylbarbituric

[193] A. Bendich, H. Getler, and G. B. Brown, *J. Biol. Chem.* **177,** 565 (1949).
[194] O. Hayaishi and A. Kornberg, *J. Am. Chem. Soc.* **73,** 2975 (1951).
[195] O. Hayaishi and A. Kornberg, *J. Biol. Chem.* **197,** 717 (1952).
[196] T. P. Wang, and J. O. Lampen, *Federation Proc.* **10,** 267 (1951).
[197] T. P. Wang and J. O. Lampen, *J. Biol. Chem.* **194,** 775 (1952).
[198] T. P. Wang and J. O. Lampen, *J. Biol. Chem.* **194,** 785 (1952).
[199] F. J. S. Lara, *J. Bacteriol.* **64,** 271 (1952).
[200] F. J. S. Lara, *J. Bacteriol.* **64,** 279 (1952).

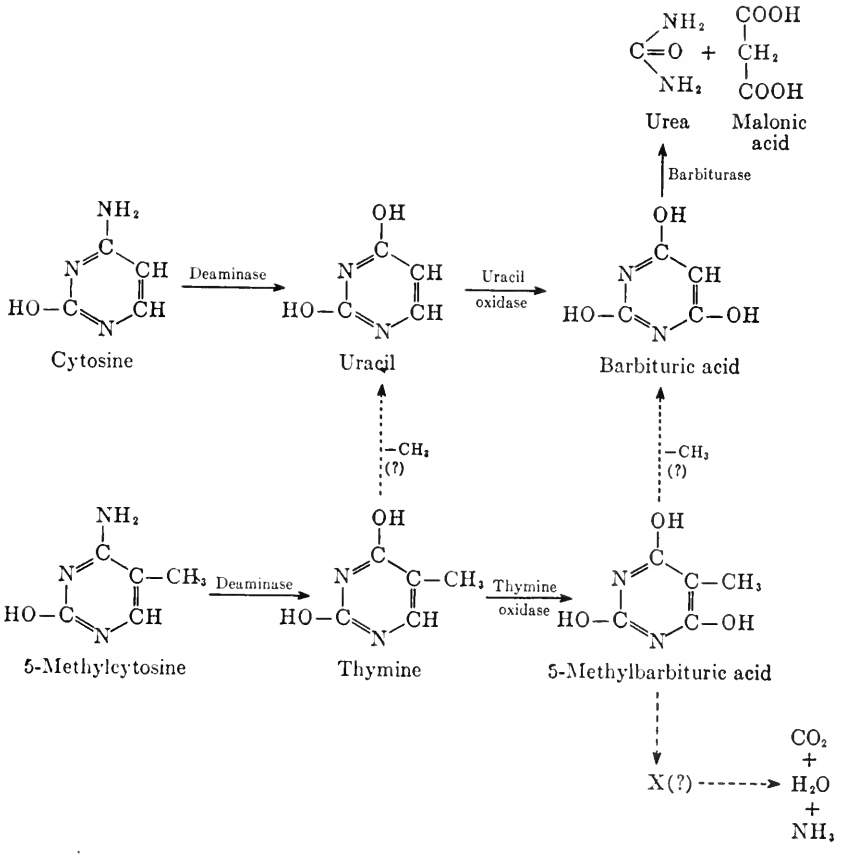

FIG. 6. The catabolism of the pyrimidines in bacteria.

acid is first demethylated to barbituric acid which is subsequently split by the barbiturase to urea and malonic acid.

VIII. Addendum

Daoust *et al.*[201] have repeated their previous studies on the rate of renewal of DNA in relation to mitosis, using lung alveolar tissue, and have confirmed that the rate of DNA synthesis calculated from results on the incorporation of P^{32} is twice that expected on the basis of new cell formation. It is again concluded that all the DNA of the two cells formed in the course of mitosis is newly synthesized. Malkin,[202] on the other hand, has found that C^{14} is taken up by the DNA of mature sea urchin sperm when incubated *in vitro* with glycine-2-C^{14} or adenine-4,6-C^{14}. Since these sperm

[201] R. Daoust, F. D. Bertalanffy, and C. P. Leblond, *J. Biol. Chem.* **207**, 405 (1954).
[202] H. M. Malkin, *Biochem. et Biophys. Acta* **12**, 585 (1953).

will not undergo further division, Malkin assumes that there is some synthesis of DNA in resting cells. The extent of C^{14} incorporation found in the sperm DNA was however small, and, bearing in mind the relatively high initial concentrations of labeled precursor used, the absolute amount of DNA synthesized must have been very minute. Taylor and McMaster[203] have followed the formation of DNA during microgametogenesis in *Lilium longiflorum* using autoradiographic methods combined with microphotometric measurements of Feulgen-stained preparations. Measurements of the DNA content of the cells studied showed it to increase in bursts at definite stages. Autoradiography of preparations grown in solutions containing P^{32} showed that the incorporation of isotope into the DNA also occurred in bursts corresponding to the stages at which the DNA content increased.

Further work has been carried out by Hokin and Hokin[204] on the uptake of P^{32} by pigeon pancreas slices *in vitro*. The assimilation of isotope by the slices was inhibited anærobically, but stimulation of amylase synthesis by the addition of amino acid mixtures led to increased labeling of the PNA nucleotides. Stimulation of secretion of the enzyme by various agents was without effect on the renewal of the PNA.

The *in vitro* incorporation of P^{32} into the PNA nucleotides of cat brain slices has been the subject of attention by Rossiter and his co-workers.[205,206] It has been shown that the isotope is assimilated in such conditions, and various factors affecting the rate of uptake of P^{32} have been studied. Thus anærobiosis, cyanide, and malononitrile effectively inhibit uptake of isotope by the nucleotides, while the addition of pyruvate, lactate, glucose, or mannose stimulates incorporation.

Lu and Winnick[207] have studied the incorporation of adenine-8-C^{14} and guanine-8-C^{14} into the nucleic acids of chick heart fibroblasts in tissue culture and have found that adenine is well utilized in the synthesis of PNA and DNA adenine and guanine. Guanine, however, was not an efficient precursor of PNA or DNA guanine and there was scarcely any conversion of guanine to polynucleotide adenine. When the cells were cultured in a medium supplemented by labeled PNA or DNA, both the PNA and DNA isolated from the cells were found to contain the isotope.

The metabolism of mouse liver nuclear and cytoplasmic PNA has been examined by Fresco and Marshak[208] using uniformly labeled adenine-C^{14}.

[203] J. H. Taylor and R. D. McMaster, *Chromosoma* **6**, 489 (1954).
[204] L. E. Hokin and M. R. Hokin, *Biochim. et Biophys. Acta* **13**, 401 (1954).
[205] H. A. Deluca, R. J. Rossiter, and K. P. Strickland, *Biochem. J.* **55**, 193 (1953).
[206] J. M. Findlay, R. J. Rossiter, and K. P. Strickland, *Biochem. J.* **55**, 200 (1953).
[207] K. H. Lu and T. Winnick, *Exptl. Cell Research* **6**, 345 (1954).
[208] J. R. Fresco and A. Marshak, *J. Biol. Chem.* **205**, 585 (1953).

These experiments have confirmed the metabolic activity of nuclear PNA as compared with cytoplasmic PNA and once more illustrate the stability of liver DNA. An extensive paper by Barnum *et al.*[209] deals with the incorporation of P^{32} into the phosphorus-containing fractions of mouse mammary carcinoma nuclei and cytoplasmic constituents at different time intervals. Their results again confirm the very rapid rate of renewal of nuclear PNA and the close metabolic similarity between the PNA's of the particulate components of the cytoplasm. In common with other workers, Barnum *et al.* have observed a distinction between the PNA of the cell sap and that of the particulate fractions. A mathematical analysis of the experimental time curves has been carried out, and the results are shown to be consistent with the conception of a common precursor of nuclear PNA, DNA, and cell sap PNA, the last of which is in turn the precursor of the PNA of the cytoplasmic particles. Moreover, their data are not consistent with the view that nuclear PNA serves as the precursor of either cell sap PNA or the PNA of the particulate components of the cytoplasm. A further pointer that cytoplasmic PNA does not necessarily arise from nuclear PNA comes from the work of Brachet and Szafarz,[210] who have examined the assimilation of orotic acid-2-C^{14} by the PNA of nucleated and enucleated portions of *Acetabularia mediterranea*. It was found over a period of months, when the two halves were incubated separately with the precursor, that the ratio of activity in the PNA of the nucleated half to that in the enucleated half remained almost constant at about 1.4.

The metabolism of orotic acid in rat liver has been examined by Hurlbert and Potter[211,212] and Hurlbert and Reichard.[213] Orotic acid is rapidly utilized in the synthesis of free uridine-5-phosphate and its various derivatives. The C^{14} content of the soluble uridine phosphate pool has been related to that of the uridylic acid of the tissue PNA at various time intervals (Fig. 7). The activities of the uridine derivatives rise very rapidly to a high level by about 3 hr. Nuclear PNA also exhibits a sharp increase in activity with a peak at about 3 hr. which is about 30 to 50% of that for the free uridine phosphate.

A survey of the uptake of P^{32} at various time intervals (2 hr. to 7 days) by the DNA's, nPNA's, and cPNA's of normal rabbit appendix, bone marrow, intestinal mucosa, kidney, spleen, and thymus has been carried

[209] C. P. Barnum, R. A. Huseby, and H. Vermund, *Cancer Research* **13,** 880 (1953).
[210] J. Brachet and D. Szafarz, *Biochem. et Biophys. Acta* **12,** 588 (1953).
[211] R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.* **209,** 1 (1954).
[212] R. B. Hurlbert and V. R. Potter, *Federation Proc.* **12,** 222 (1953).
[213] R. B. Hurlbert and P. Reichard, *Acta Chem. Scand.* **8,** 701 (1954).

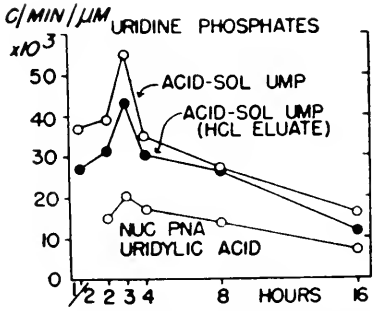

FIG. 7. Specific activities of uridine phosphates from the acid-soluble fraction and from the nPNA of rat liver at different times after the administration of orotic acid-6-C^{14}. "Acid-soluble UMP" is the free uridine-5'-monophosphate of the acid extract. "Acid-soluble UMP (HCl eluate)" represents the activity of the uridine derivatives obtained by hydrolysis of the mixture of uridine compounds which are eluted from the ion-exchange column with HCl (Hurlbert and Potter[211]).

out by Smellie *et al.*[214,215] These tissues fall into three categories according to their relative rates of DNA turnover:

1. Kidney in which the renewal of DNA is very slight.

2. Appendix and bone marrow in which the DNA turnover is very rapid, being greater than that of the cPNA and only slightly lower than that of the nPNA.

3. Intestinal mucosa, spleen, and thymus where the DNA is replaced at a rate intermediate between that of kidney and bone marrow and lower than either nPNA or cPNA.

In all the tissues examined, the activities of the nPNA's rose very sharply, reaching maxima earlier and higher than the corresponding DNA's and cPNA's. The turnover of appendix and bone marrow cPNA was also rapid, being only slightly lower than that of the DNA's; in the other tissues, however, cPNA renewal was more moderate.

Klein and Forssberg[216] have examined the effects of X-irradiation on the metabolism of the nucleic acids in Ehrlich mouse ascites tumor. A dose of 1250 r. of X-rays completely inhibited mitosis over a period of 16 hr. without producing any increase in cell death rate. In these conditions a linear increase in cell volume occurred with time and the increase in PNA per cell corresponded to the increase in volume. On the other hand, the DNA per cell was raised only slightly. Further studies using glycine-2-C^{14} [217]

[214] R. M. S. Smellie, G. F. Humphrey, E. R. M. Kay, and J. N. Davidson, *Biochem. J.* **58**, xxxvii (1954).

[215] R. M. S. Smellie, G. F. Humphrey, E. R. M. Kay, and J. N. Davidson, *Biochem. J.* in press 1955.

[216] G. Klein and A. Forssberg, *Exptl. Cell Research* **6**, 211 (1954).

[217] A. Forssberg and G. Klein, *Exptl. Cell Research*, **7**, 480 (1954).

indicated a drop of about 30% in the uptake of isotope by both PNA and DNA of the irradiated cells as compared with the controls. There is therefore a contrast between the results obtained by purely chemical analysis and those from the isotope measurements since with DNA the chemical method indicates a fall in the rate of DNA synthesis after irradiation to about 33% of that in the controls, while the isotope method suggests that DNA synthesis falls to about 70% of the control level. Similarly, chemical measurements with PNA indicate an undiminished rate of synthesis after irradiation, while isotope methods show a fall in renewal of PNA to about 70% of the control value.

The effect of X-irradiation (1000 r.) of rabbit abdominal viscera and femora on the blood cell population and on the uptake of P^{32} by the DNA, nPNA, and cPNA of appendix, bone marrow, kidney, and thymus has been studied by Smellie *et al.*[214,215] Although the erythrocyte pattern was not greatly altered until about 49 hr. after irradiation, the proportion of lymphocytes fell rapidly within 2 hr. of treatment and there was an initial sharp increase in the granulocytes.

DNA synthesis in appendix and bone marrow was reduced to about 35% and 65%, respectively, of the normal level within 2 hr. of exposure to the X-rays, the values at 20 hr. being 17% and 23% and at 49 hr. 33% and 76%. In thymus DNA turnover was not affected at 2 hr. but by 20 hr. was reduced to 37% of the normal, recovering by 49 hr. to 46%.

The activities of the nPNA and cPNA of appendix and bone marrow were depressed within 2 hr. of irradiation and remained low even after 49 hr. In kidney both nPNA and cPNA showed slightly elevated activities 2 hr. after irradiation but by 20 hr. and 49 hr. the values were below normal. Thymus nPNA and cPNA were little affected by 2 hr. but fell markedly 20 hr. and 49 hr. after exposure to the X-rays.

The incorporation of P^{32} into the DNA, nPNA, and cPNA of appendix, bone marrow, spleen, and thymus of rabbits which had been treated with phenylhydrazine to induce hemolytic anemia and hyperplasia of the bone marrow has been studied.[214,215] The activity of bone marrow DNA was increased to approximately twice the control value, while in spleen a tenfold increase in activity was recorded. The cPNA of bone marrow and spleen exhibits a considerable increase in activity while the turnover of nPNA is also slightly elevated. The nucleic acids of appendix and thymus were unchanged in the anemic animals.

In the field of bacterial viruses, several extensive review articles have appeared in recent months: Putnam,[218] Cohen,[219] Hershey,[220] and Kozloff.[221]

[218] F. W. Putnam, *Advances in Protein Chem.* **8**, 175 (1953).
[219] S. S. Cohen, *Cold Spring Harbor Symposia Quant. Biol.* **18**, 221 (1953).
[220] A. D. Hershey, *Cold Spring Harbor Symposia Quant. Biol.* **18**, 135 (1953).

Cohen and Weed[222] have examined the synthesis of 5-hydroxymethylcytosine by T_6r^+ bacteriophage using orotic acid-2-C^{14} and serine-3-C^{14}. These experiments showed that the hydroxymethyl group of the virus hydroxymethylcytosine was almost certainly not derived from the methyl group of the *E. coli* DNA thymine. Much of the hydroxymethylcytosine of the virus DNA arose from small-molecule precursors in the medium, and it was found that host cytosine but not thymine contributed to the formation of virus hydroxymethylcytosine.

The catabolism of uracil in the rat has been studied by Rutman *et al.*[223] The direct formation of urea as the most important degradative pathway for uracil in rats has been eliminated. At endogenous levels of uracil metabolism negligible quantities of urea are formed, while at higher levels of uracil metabolism some urea is formed.

A comprehensive review by Schulman[224] of purine and pyrimidine anabolism and catabolism has recently appeared.

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[221] L. M. Kozloff, *Cold Spring Harbor Symposia Quant. Biol.* **18,** 209 (1953).

[222] S. S. Cohen and L. L. Weed, *J. Biol. Chem.* **209,** 789 (1954).

[223] R. J. Rutman, A. Cantarow, and K. E. Paschkiss, *J. Biol. Chem.* **210,** 321 (1954).

[224] M. P. Schulman, *in* "Chemical Pathways of Metabolism" (Greenberg, ed.), Vol. 2, p. 223. Academic Press, New York, 1954.

CHAPTER 27

The Biological Role of the Deoxypentose Nucleic Acids

ROLLIN D. HOTCHKISS

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I. Deoxypentose Nucleic Acids as Genetic Determinants

The possibility that the deoxynucleic acids, in the form of chromatin, have a role in genetic mechanisms has been suggested implicitly, and

often explicitly, from relatively early times. It was almost inevitable that chromatin should speculatively be assigned such a function as soon as the processes in which it undergoes such dramatic change—mitosis, meiosis, and fertilization—came to be considered as genetic ones. This proposition, expressed during the last half century progressively more precisely and significantly, is now supported by certain evidences of cause and effect which point up the general importance of the supposed relationship. Let us first consider the circumstantial evidence supplied by the study of DNA (deoxypentosenucleic acid) distribution in tissues—a subject already treated in Chapters 18 and 19—then proceed to discuss the evidences of a dynamic functional relationship of DNA to genetic processes.

1. Organization of DNA in Genetic Elements of Tissues

Three lines of investigation—chemical, cytological, and genetic—had to merge to furnish the information we can now marshal to support the genetic significance of DNA. Chemists for many years had been learning the properties of this material as it was recovered from separated nuclei, from sperm heads, and from a variety of tissues. On another side, its histological analog, chromatin, was being recognized *in situ* in nuclei, and the intricate behavior of the chromosomes at mitosis was being observed. Meanwhile, too, the conception of the genes, units transmitting heritable properties from cell to cell, was being developed. It was the integration at the borderlines between the three fields of chemistry, cytology, and genetics which in retrospect seems to have provided the greatest or most difficult steps in the development which we are discussing.

a. Localization of DNA within the Cell

In 1922, Feulgen made the advance which permitted a correlation between the chemical and cytological information which had accumulated.[1] He had shown, ten years before, that the instability of DNA in warm acid would result in the liberation of aldehyde groups giving color with Schiff's reagent. This characteristic and specific reaction, demonstrable with isolated purified material, furnished precision to cytological studies. The acidic components of the chromatin, which bind basic dyes, could now be identified as DNA nucleoproteins, rather than PNA (ribonucleic acid, pentosenucleic acid), phosphoprotein, etc. Not only is DNA responsible for the characteristic chromogenicity of chromatin, but subsequent work, with the histological, enzymic, and photometric methods since developed, has revealed that virtually all of the DNA of the cell is localized in the nuclear chromatin. These investigations have been covered in Chapters 17, 18, and 19.

[1] R. Feulgen and H. Rossenbeck, *Z. physiol. Chem.* **135,** 203 (1924).

Another cytochemical correlation, different in its nature, provides supporting evidence. In 1942, Mirsky and Pollister[2] identified the fibrous protein extractable from tissues with strong salt solutions as DNA-protein, and showed that its extraction from cytological preparations occasioned the loss of definition and stainability of the nuclear material.

Cytochemical evidence then, relative to DNA function, has taught us that DNA is a characteristic constitutent of the chromosomes and all of the cellular DNA is probably associated with these bodies.

b. Localization of Genetic Determinants within the Cell

In another boundary science, parallels were being drawn; in this case between cytological phenomena and genetic processes. Even for the early observers, the behavior of the sperm nucleus in fertilization of the egg was a reasonable indication that the nucleus was probably the essential contribution from the sperm and therefore the source of the inheritable factors of the paternal line. The properties of denucleated cells or parthogenetically induced ova were sufficiently in keeping with this conception to support it.

Of course, it is not reasonable to assume, a priori, that the relatively easily observable chromatin bodies are more important than some invisible elements in the functions of the nucleus. It was not until the classic papers of Morgan and his co-workers[3,4] pointed out the impressive parallels between the organized chromosome movements and the movements of the genetic determinants deduced from Mendelian genetics, that the chromosome was finally accepted as a "gene carrier." Among the most significant parallels in this demonstration were: (1) the number of homologous chromosomes carried was the same as the number of genetic units or groups, especially with regard to the sex-linked groups; (2) correspondence in the cycles of reduction, duplication, or pairing of these respective cytological and genetic entities in the reproduction of various species; and (3) the genetic effects of the chromosome redistribution when a pair failed to divide (nondisjunction).[5] Subsequently, other important correlations have been shown, particularly the genetic results accompanying translocations of chromosome fragments or breakage of the chromosomes either by X-rays[6] or by occasional spontaneous processes. It is clear that the Mendelian genes are associated in some manner with the chromosomes or equivalent structures.

Cytochemical data, then, has located DNA in the same cellular struc-

[2] A. E. Mirsky and A. W. Pollister, *Proc. Natl. Acad. Sci. U.S.* **28,** 344 (1942).
[3] T. H. Morgan, *J. Exptl. Zool.* **11,** 365 (1911).
[4] A. H. Sturtevant, *J. Exptl. Zool.* **14,** 43 (1913).
[5] C. B. Bridges, *Genetics* **1,** 1, 107 (1916).
[6] H. J. Muller, *Science* **66,** 84 (1927).

tures, the chromosomes, as those the cytogeneticists have shown must carry the genes.[7]

c. Quantitative Cytochemical Regularities in DNA Distribution

The suggestion that DNA might be the genetically active material of the cell has thus been supported to the extent that localization is at least appropriate to this function. The recognition that chromosomes have constituents other than DNA, especially histones and other proteins, however, made it impossible to claim more than this.

Quantitative cytochemical studies, stimulated in large part by the bacterial transformations to be discussed in the next section, have recently made a genetic function of DNA seem somewhat more convincing. Boivin *et al.*[8] showed that the DNA satisfied the additional criteria of being essentially constant in amount in the (diploid) somatic nuclei of various tissues within a species, and of being present in half quantities in the (haploid) sperm cells (Chapter 19). Mirsky and Ris[9] confirmed this relationship in general, extending the study to a number of new species. Mirsky[10] has furnished the additional information that the various nuclear proteins (histone, "residual protein" of the chromosomes, nuclear enzymes) did not—at least as defined by the analytical methods used—have the distribution expected for gene material, and exhibited by the DNA.

Differences in the DNA content of individual nuclei of rat liver[11-13] showed three levels of DNA in ratios 1:2:4, corresponding to three sizes of nucleus, presumably representing diploid, tetraploid, and octaploid nuclei. Ogur *et al.*[14] have analyzed yeast strains varying from haploid to tetraploid and have reported that DNA content is more consistently in direct relation to the degree of ploidy than dry weight, PNA, or metaphosphate content. Smaller differences, within a size class, between indi-

[7] It is generally believed that the chromosomes maintain their essential DNA localization and genetic integrity during the mitotic interphase, although they are diffuse and difficult to observe at this stage. In any case, the correlation between DNA distribution and cytogenetic organization is applicable during the more active nuclear stages, and the quantitative data to be discussed tends to show that there is no gross discontinuity at interphase.

[8] A. Boivin, R. Vendrely, and C. Vendrely, *Compt. rend.* **226,** 1061 (1948).

[9] A. E. Mirsky and H. Ris, *Nature* **163,** 666 (1949).

[10] A. E. Mirsky, *in* "Genetics in the 20th Century" (Dunn, ed.). Macmillan, New York, 1951.

[11] H. Ris and A. E. Mirsky, *J. Gen. Physiol.* **33,** 125 (1949).

[12] H. H. Swift, *Physiol. Zool.* **23,** 169 (1950).

[13] C. Leuchtenberger, R. Vendrely, and C. Vendrely, *Proc. Natl. Acad. Sci. U.S.* **37,** 33 (1951).

[14] M. Ogur, S. Minckler, G. Lindegren, and C. C. Lindegren, *Arch. Biochem. and Biophys.* **40,** 175 (1952).

vidual nuclei[15,16] have been variously interpreted as meaning that DNA distribution is altered during tissue differentiation, or that the amount of DNA per nucleus actually fluctuates about an average value, as evidences of stages in chromosome duplication, or simply of experimental error (Chapter 19).

The elegant ultraviolet microspectrophotometric methods of Caspersson allowed this worker and Schultz to observe that certain genetic position effects in *Drosophila* could be correlated with cytochemical changes in the nucleic acid absorption at the corresponding region of the giant salivary gland chromosomes.[17]

The amounts of DNA found cytochemically to be exclusively associated with the chromosomes, therefore, are appropriate for a genetic function. A genetic role of DNA is a satisfactory assumption but is not a necessary one on this basis. We have considered so far the indications provided from cytochemistry and cytogenetics, two of the borderline fields relating the three primary sciences of chemistry, cytology, and genetics. A more direct and convincing kind of evidence will next be considered—that supplied from the third borderline field, the one concerned with both chemistry and genetics.

2. Evidences of Genetic Functioning of DNA

It is in the realm of genetic chemistry[18] that we find cause and effect information—direct evidence that this or that chemical substance is involved in, or modifies, elementary genetic processes. This approach was being developed more or less simultaneously with the cytochemical one during the last two or three decades.

a. Mutational Effects by Agents Reacting with DNA

Indication of *in vivo* genetic action attributable to DNA in intact cells is furnished by the relatively specific agent, ultraviolet radiation. The variation with wavelength (action spectrum) of the effectiveness for

[15] L. Lison and J. Pasteels, *Arch. Biol. (Liège)* **62,** 1 (1951).

[16] B. C. Moore, *Chromosoma* **4,** 563 (1952).

[17] J. Schultz and T. Caspersson, *Arch. exptl. Zellforsch. Gewebezücht.* **22,** 650 (1939).

[18] The term genetic chemistry is used here to signify particularly the chemistry of genetic processes, somewhat in distinction to the term chemical genetics. Although Beadle clearly conceived the latter to include the whole chemical relation between genetic determinants and their end-effects (G. W. Beadle, *in* "Genetics in the 20th Century" (Dunn, ed.), p. 221. The Macmillan Co., New York, 1951, the terms chemical (and biochemical) genetics have come in practice to be associated with a large body of data in which it is primarily intermediary biochemistry that is illuminated. At the present moment the biochemical characters unfortunately seem hardly more closely related to the gene than the previously studied morphological ones.

producing genic mutations in fungi[19] and corn [20] were shown to be equivalent to an ultraviolet absorption curve of nucleic acid. The specificity of this indication is not great enough to show unquestionably that protein is not absorbing the effective quanta, and mutagenic action is indeed considerable at the absorption maximum for protein. Furthermore, even though the ultraviolet energy may have been absorbed by nucleic acid or nucleoprotein, activated molecules or atoms may then have produced the ultimate significant chemical changes at some site other than the nucleic acid itself. These conceptions, and the recognition that added substances and environmental factors could considerably modify the number of mutations detected after ultraviolet irradiation, tended to prevent any general acceptance of this phenomenon as an indication of the chemical nature of genes. More recently, the possibility of reversing ultraviolet mutational and lethal effects by subsequent exposure to visible light[21,22] (photoreactivation) as much as three hours later has emphasized that mutagenesis by this agency is an indirect and certainly not a simple process. Nevertheless, the known facts fit well with a genetic role for DNA. Prolonged ultraviolet irradiation of high-molecular calf thymus DNA can bring about structural changes interpretable as depolymerization.[23] Treatment of *Escherichia coli* with low doses of ultraviolet inhibits DNA synthesis in these bacteria without appreciably affecting respiration or PNA synthesis, and does this before an effect upon growth is apparent.[24]

Some evidences of the chemical nature, or at least reactivity, of gene material can be found in the demonstrated chemical effects of certain other mutagenic agents. Sulfur mustard probably was the first chemical substance clearly shown to induce mutations.[25] On the basis of the kinetics of inactivation by sulfur mustard of a diverse series of biological materials ranging from pure protein enzymes to several viruses, bacteria, and yeast, Herriott concluded that DNA was a much more sensitive component of living cells than protein or PNA.[26] This conclusion, arrived at by biological test, seems significant in view of the numerous reports that sulfur and nitrogen mustards can produce mutations in a number of species. The demonstration that these substances can react chemically with DNA has

[19] A. Hollaender and C. W. Emmons, *Cold Spring Harbor Symposia Quant. Biol.* **9,** 179 (1941).
[20] L. J. Stadler and F. M. Uber, *Genetics* **27,** 84 (1942).
[21] A. Kelner, *J. Bacteriol.* **58,** 511 (1949).
[22] A. Novick and L. Szilard, *Proc. Natl. Acad. Sci. U.S.* **35,** 591 (1949).
[23] A. Hollaender, J. P. Greenstein, and W. V. Jenrette, *J. Natl. Cancer Inst.* **2,** 23 (1941).
[24] A. Kelner, *J. Bacteriol.* **65,** 252 (1953).
[25] C. Auerbach and J. M. Robson, *Nature* **157,** 302 (1946).
[26] R. M. Herriott, *J. Gen. Physiol.* **32,** 221 (1948).

generally required higher concentrations and other favorable circumstances, so that the milder conditions of the biological experiments are not met. However that may be, mustards in high concentrations react avidly with the phosphate and amino groups of nucleic acids, producing substituted ("alkylated") derivatives. Under relatively milder conditions, they initiate processes leading to loss of viscosity and apparent depolymerization.[27-29] Their ability to precipitate DNA-protein has also been noted; their interaction with DNA nucleoproteins is greater than with other proteins.[30,31] Mustard derivatives having only a single reactive halogen group have been shown to produce both mutations[32] and depolymerization of DNA.[29] Since these cannot produce cross-linkages between proteins or other substances, one alternate hypothesis relating mutagenesis to the chemical potentialities of the mustards is made unlikely. It has also been reported that mustard treatment causes a block in the synthesis of DNA, but not in that of PNA, both in the amphibian embryo and in *E. coli*.[33,34]

The sulfur and nitrogen mustards are in general highly toxic for living cells, and they produce chromosome breakages and other effects—these may or may not be extreme manifestations of the processes responsible for mutagenesis (e.g., lethal mutations). It is not usually clear, however, which of these types or degrees of biological effect should be considered parallel with the processes being investigated in the test-tube. What does seem to remain true is that DNA's are among the substances most highly susceptible to the chemical action of the mutagenic mustards.

Other mutagenic substances do not as yet furnish evidence suggesting, even so loosely as this, a cause-and-effect relationship between reactivity with nucleic acids and mutagenesis. Acriflavin is known to be capable of precipitating with nucleic acids and nucleotides[35] (so do many other bases), and this interaction has been proposed as an explanation for its growth-inhibiting effects.[36] The actions of this and related acridines upon genetic systems include mutagenic effect in bacteria,[37] destruction of particle-

[27] J. A. V. Butler, L. A. Gilbert, and K. A. Smith, *Nature* **165,** 714 (1950).
[28] E. C. Gjessing and A. Chanutin, *Cancer Research* **6,** 593 (1946).
[29] J. A. V. Butler and K. A. Smith, *J. Chem. Soc.* **1950,** 3411.
[30] I. Berenblum and R. Schoental, *Nature* **159,** 727 (1947).
[31] T. E. Banks, J. C. Boursnell, G. E. Francis, F. L. Hopwood, and A. Wormall, *Biochem. J.* **40,** 745 (1946).
[32] C. M. Stevens, A. Mylroie, C. Auerbach, H. Moser, K. A. Jensen, I. Kirk, and M. Westergaard, *Nature* **166,** 1019, 1020 (1950).
[33] D. Bodenstein and A. A. Kondritzer, *J. Exptl. Zool.* **107,** 109 (1948).
[34] R. M. Herriott, *J. Gen. Physiol.* **34,** 761 (1951).
[35] H. McIlwain, *Biochem. J.* **35,** 1311 (1941).
[36] L. Massart, G. Peeters, J. de Ley, R. Vercauteren, and A. van Houcke, *Experientia* **3,** 288 (1947).
[37] E. M. Witkin, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 256 (1947).

borne cytochrome systems of yeast,[38] and partial disruption of bacteriophage formation,[39] but it is not clear whether such effects will prove to be widespread or limited to a few species.

One group of mutagenic agents seem to be metabolically related to the nucleic acids. Such methylated purines as caffeine and theophylline are effective mutagens for *E. coli* although not highly toxic for these organisms.[40] The possibility that these bases might interfere with normal purine incorporation seems somewhat substantiated by the finding that nucleosides of the natural bases counteract the mutagenesis.[41] There are some reasons to relate the mutagenic capacity of another agent of slight toxicity, manganous ion,[42] with its tendency to react with DNA.[43]

It is known that X-radiation, a notable mutation-producing agent, results in the production of such active intermediates as peroxides, and H and OH radicals. It is therefore of interest that X-rays and some of these by-products have disruptive effects upon DNA preparations. Isolated calf thymus DNA becomes progressively less viscous (gradually depolymerized) after X-irradiation[44-47] or treatment with peroxides or OH radicals.[48,49] There may also be chemical degradation.[50] Not all forms of DNA investigated have been shown to be degraded after X-irradiation, possibly because the effectiveness of active radical formation is dependent upon the presence of oxygen and water. Precipitated anhydrous DNA,[47] and the *in situ* material of thymus nuclei (citric acid treated)[51] or plant buds[52] were not apparently affected. On the other hand, irradiation of the intact rat thymus,[47] or of nucleated erythrocytes,[53] did lead to depolymeri-

[38] B. Ephrussi, H. Hottinguer, and A.- M. Chimenes, *Ann. inst. Pasteur* **76,** 351 (1949); P. P. Slonimski and B. Ephrussi, *ibid.* **77,** 47 (1949).

[39] R. I. de Mars, S. E. Luria, H. Fisher, and C. Levinthal, *Ann. inst. Pasteur* **84,** 113 (1953).

[40] A. Novick and L. Szilard, *Proc. Natl. Acad. Sci. U. S.* **36,** 708 (1950).

[41] A. Novick and L. Szilard, *Nature* **170,** 926 (1952).

[42] M. Demerec and J. Hanson, *Cold Spring Harbor Symposia Quant. Biol.* **16,** 215 (1951).

[43] R. B. Roberts and E. Aldous, *Cold Spring Harbor Symposia Quant. Biol.* **16,** 229 (1951).

[44] A. H. Sparrow and F. M. Rosenfeld, *Science* **104,** 245 (1946).

[45] B. Taylor, J. P. Greenstein, and A. Hollaender, *Arch. Biochem.* **16,** 19 (1948).

[46] G. C. Butler, *Can. J. Research* **27B,** 972 (1949).

[47] G. Limperos and W. A. Mosher, *Am. J. Roentgenol. Radium Therapy* **63,** 681 (1950).

[48] W. von B. Robertson, M. W. Ropes, and W. Bauer, *Proc. Soc. Exptl. Biol. Med.* **49,** 697 (1942).

[49] J. A. V. Butler and K. A. Smith, *Nature* **165,** 847 (1950).

[50] G. Scholes and J. Weiss, *Exptl. Cell Research* Suppl. 2, 219 (1952).

[51] H. von Euler and L. Hahn, *Acta Radiol.* **27,** 269 (1946).

[52] M. J. Moses, R. J. Dubow, and A. H. Sparrow, *J. Natl. Cancer Inst.* **12,** 232 (1951).

[53] M. Errera, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 60 (1947).

zation of the DNA. One of the biochemical systems most sensitive to X-rays in tissues appears to be the DNA synthesizing system[54-58] (see Chapter 26).

At the cytogenetic level, the sulfur and nitrogen mustards and X-rays produce a variety of major and minor breakages and rearrangements of the chromosomes, involving loss and refusion of chromosome strands, etc. It may hardly be expected that a single mechanism like the depolymerization of DNA lies behind all of these effects, but the vulnerability of DNA to these agents is a striking fact that surely has some relation to their mutagenic effects.

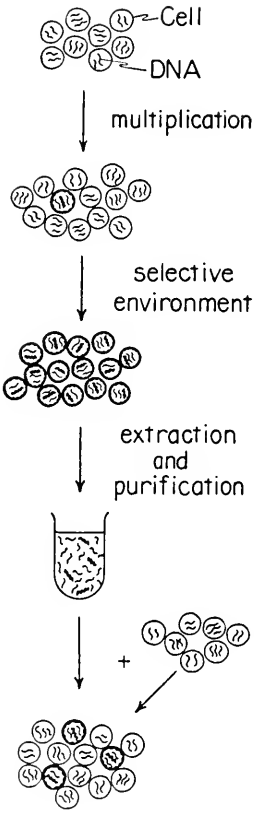
b. Bacterial Transformations—Observed Genetic Action of Isolated DNA

The cytochemical studies of the DNA content of nuclei and the chemical studies with mutagenic agents were carried out during the last decade, when such studies were stimulated and influenced by the important finding that isolated bacterial DNA can have a genetic effect upon bacterial cells.

In 1944, Avery *et al.* reported that DNA extracted from an encapsulated ("smooth"; S) strain of pneumococcus would transform unencapsulated ("rough" colony type; R) cells into encapsulated cells.[59] There were three especially significant features about these transformations: (1) The capsule of the newly modified cells was always constituted of the same serologically type-specific polysaccharide as that of the strain from which the DNA was derived. (2) The conversion could be brought about in a rough pneumococcal strain which was never observed to change or mutate spontaneously into that smooth type or any other. (3) The smooth cells produced would propagate indefinitely as an encapsulated strain without further exposure to the DNA—in fact, would themselves go on to produce unlimited quantities of DNA with the same potentialities. See Fig. 1.

It was clear from this work that the pneumococcal DNA preparation had performed two functions usually attributed to genes: it had induced a specific inheritable property (capsule synthesis), and it had initiated its own reduplication (formation of more DNA with the same activity) as well. Although only one single category of character in a single species was involved, the genetic implications of these findings were not overlooked and have had a profound effect in orienting much subsequent research in a number of related fields. The original authors principally concentrated their attention on the nature and properties of the transforming agents

[54] J. S. Mitchell, *Brit. J. Exptl. Pathol.* **23**, 309 (1942).
[55] H. von Euler and G. von Hevesy, *Arkiv Kemi* **17A**, No. 30, 1 (1944).
[56] B. E. Holmes, *Brit. J. Radiol.* **22**, 487 (1949).
[57] G. Hevesy, *Nature* **163**, 869 (1949).
[58] R. Abrams, *Arch. Biochem. and Biophys.* **30**, 90 (1951).
[59] O. T. Avery, C. M. MacLeod, and M. McCarty, *J. Exptl. Med.* **79**, 137 (1944).



Parent culture

Culture + rare mutant, (e.g. drug resistant, etc., 1 in 10^7)

Mutant culture (or stock variant, e.g. encapsulated)

Purified mutant DNA (transforming agent)

+

Parent culture

↓

Culture containing transformed cells (1 in 10^2 to 10^3)

FIG. 1. Transformation of a bacterial culture.

themselves. Other developments since have revealed a number of other characters to be transformable in pneumococci and in other bacteria species, and have also been concerned with the relation of the DNA factors to normal genetic mechanisms.

c. Chemical Nature of Bacterial Transforming Agents

Historically, the first source of pneumococcal transforming factor was heat-killed encapsulated cells, which were shown to act, first in the infected mouse[60] and later in the test-tube,[61] upon unencapsulated cells. The ultimate recognition of the activity of a DNA[59] came after the successful preparation of the agent in a cell-free form by Alloway.[62]

[60] F. Griffith, *J. Hyg.* **27,** 113 (1928).

[61] M. H. Dawson, *J. Exptl. Med.* **51,** 123 (1930); M. H. Dawson and R. H. P. Sia, *ibid.* **54,** 681 (1931).

[62] J. L. Alloway, *J. Exptl. Med.* **55,** 91 (1932); **57,** 265 (1933).

TABLE I

PROPERTIES OF PNEUMOCOCCUS TYPE III TRANSFORMING AGENT

Property	Pneumococcus-transforming agent	Calf thymus DNA	Ref.
Carbon content, %	34.3–35.5	34.2 "theory"	59
Hydrogen content, %	3.8- 3.9	3.2 "theory"	59
Nitrogen content, %	13.4–15.9	15.3 "theory"	59
Phosphorus %	8.5- 9.1	9.05 "theory"	59
Nitrogen/phosphorus	1.6- 1.75	1.69 "theory"	59
	1.72	1.72 found	63
Phosphorus/ultraviolet absorption at 260 mμ	4.4[a]	4.2[a]	63
Pentose (Bial reaction)	Slight	Slight	59
Deoxypentose (Dische)	Present	Present	59
Deoxypentose/ultraviolet absorption at 260 mμ	0.086[a]	0.093[a]	63
Bases found	Thymine, adenine, guanine, cytosine (no uracil)	Thymine, adenine, guanine, cytosine, 5-methylcytosine?	63
Thymine/P atom	0.292	0.280	64
Adenine/P atom	0.276	0.276	64
Guanine/P atom	0.190	0.235	64
Cytosine/P atom	0.167	0.197	64
Ultraviolet absorption:			
Maximum, mμ	260	(260)	59
Minimum, mμ	*ca.* 235	(235)	59
Amino acid in hydrolysate, as % of total N	0.86[b]	1.1[b]	63
Glycine (from adenine), as % of total amino acid	100 ± 2		65
Protein, %	Less than 0.02		65
Specific carbohydrate	Faint trace		59

[a] Based on relative optical density units.

[b] Comparative values at time when protein hydrolysis is complete; release here is slow, linear, dependent upon acidity and temperature.

(1) *Recognition of DNA Nature.* The purified pneumococcus-transforming factor inducing capsular Type III has the chemical and analytical properties of a DNA, as indicated in Table I. It will be noted that it is

[63] R. D. Hotchkiss, *Colloq. intern. centre natl. recherche sci. (Paris)* **8**, *Unités biol. douées contin. génét.* 57 (1949).

[64] M. M. Daly, V. G. Allfrey, and A. E. Mirsky, *J. Gen. Physiol.* **33**, 497 (1950).

[65] R. D. Hotchkiss, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 426. The Johns Hopkins Press, Baltimore, 1952.

essentially a deoxypentose polynucleotide containing the usual four bases and lacking uracil.

Physically, the transforming preparations appear to consist largely of asymmetric particles giving, like thymus DNA, highly viscous aqueous solutions, and behaving similarly to this material in the ultracentrifuge. Their biological activity is destroyed, and viscosity reduced, by deoxyribonuclease.[66] Preparations of this enzyme from streptococci, and crystallized pancreatic preparations, which do not have any other recognized enzymic activity, in very small amounts rapidly inactivate the transforming agent.[63, 67] Significant also is the fact that of a number of tissue, serum, and heated serum preparations, only those which contained active deoxyribonuclease were able to bring about this inactivation.[59] Ribonuclease, trypsin, and chymotrypsin had no effect upon activity. Certain parallels have also been drawn between the effects of heat and pH change upon viscosity and upon biological activity.[67]

(2) *Evidence that Other Substances Are Not Present.* These results demonstrated that the transforming preparation was essentially a DNA preparation and that its biological activity was dependent upon the intactness of high-molecular DNA which it contained. Although such extracts have detectable transforming activity at concentrations as low as a few thousandths of a microgram per milliliter, these findings do not point unequivocally to identity of the DNA and the transforming factor. One might suppose that (1) only a part of the DNA is biologically functional, or possibly that (2) a small, still more highly active fraction of the material consists of carbohydrate[68] or protein,[69] combined with, or stabilized by, DNA. The serological tests originally applied to the material should have given a barely recognizable positive reaction if there had been around 5% of a serologically active protein, or 0.05% of type-specific carbohydrate present.[59]

The generally accepted—though still largely unexplained—biological specificities of protein made it seem most essential to demonstrate, if possible, that the biologically specific transforming activity was not attributable to protein, e.g., to a trace of nucleoprotein. The evidence against a major content of protein includes the following facts: (1) the bulk of the pneumococcal protein, at least, is removed during purification without loss of the transforming activity,[63, 70] (2) as mentioned, the product does not

[66] M. McCarty and O. T. Avery, *J. Exptl. Med.* **83,** 89 (1946).

[67] S. Zamenhof, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 301. Johns Hopkins Press, Baltimore, 1952.

[68] M. Stacey, *in* "The Nature of the Bacterial Surface" (Miles and Pirie, eds.), p. 29. Blackwell Scientific Publications, Oxford, 1949.

[69] A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.* **30,** 117 (1946).

[70] S. Zamenhof, G. Leidy, H. E. Alexander, P. L. FitzGerald, and E. Chargaff, *Arch. Biochem. and Biophys.* **40,** 50 (1952).

give serological reaction of proteins, nor (3) give qualitative protein color reactions, nor (4) have the nitrogen–phosphorus ratio of a nucleoprotein.[59] Evidences that not even very small amounts of biologically significant protein are present in the purified agent are (5) the failure of trypsin and chymotrypsin to inactivate the transforming agent,[59] and (6) the fact that all of the amino acid detectable in acid hydrolysates is quantitatively accounted for as glycine,[65] and that (7) the rate at which this amino acid appears in the hydrolysate is the same as that of the slow linear degradation of adenine to glycine, rather than that of the initially rapid, then diminishing, hydrolysis of a protein or peptide.[63] The last two findings allowed the estimate that protein, if present at all, constituted not more than 0.02% of the purified transforming preparation. These results do not of course furnish any obstacle to the view that the transforming DNA may interact with preexisting or newly formed protein material after it enters a recipient cell. Furthermore, as will be indicated later, probably only a small part of the DNA present is involved in transformations of any one particular trait.

The absence of protein, as just described, together with the fact that the biological activity is associated with a single homogeneous boundary in both the ultracentrifuge[59] and the electrophoretic cell[59,70] shows that the isolated transforming agent is not associated with a virus particle, chromosome fragment, or with cellular debris. The inactivation by crystallized deoxyribonuclease does not result from the release of inhibitors but from actual destruction of the high-molecular deoxynucleate material.[65]

So far as is known, these conclusions apply to all of the bacterial transforming agents to be discussed in the next section. All have been shown to be inactivated by purified deoxynuclease and some of them have been prepared in a form relatively free of protein and ribonucleic acid.[59,63,65,70,71,75,77,82]

d. Instances of Bacterial Transformation by DNA

Since the demonstration of the transfer of the Type III capsular character in pneumococci, a number of other properties have been experimentally

[71] M. McCarty and O. T. Avery, *J. Exptl. Med.* **83,** 97 (1946).
[72] R. Austrian, *Bull. Johns Hopkins Hosp.* **90,** 170 (1952).
[73] R. Austrian, *Bull. Johns Hopkins Hosp.* **91,** 189 (1952).
[74] A. Boivin, A. Delaunay, R. Vendrely, and Y. Lehoult, *Experientia* **1,** 334 (1945).
[75] H. E. Alexander and G. Leidy, *J. Exptl. Med.* **93,** 345 (1951).
[76] H. E. Alexander and W. Redman, *J. Exptl. Med.* **97,** 797 (1953).
[77] R. D. Hotchkiss, *Cold Spring Harbor Symposia Quant. Biol.* **16,** 457 (1951).
[78] R. Austrian and M. S. Colowick, *Bull. Johns Hopkins Hosp.* **92,** 375 (1953).
[79] R. D. Hotchkiss and J. Marmur, *Proc. Natl. Acad. Sci. U. S.* **40,** 55 (1954).
[80] D. T. Klein and R. M. Klein, *J. Bacteriol.* **66,** 220 (1953).
[81] C. M. MacLeod and M. R. Krauss, *J. Exptl. Med.* **86,** 439 (1947).
[82] H. E. Taylor, *J. Exptl. Med.* **89,** 399 (1949).

induced in like manner. Each significant case presents a specific trait induced only by DNA coming from a strain already possessing that specific property. Other capsule types were induced in pneumococci.[71-73] then analogously in *E. coli*[74] and in *Hemophilus influenzae*,[75] and subsequently in meningococci.[76] Drug resistance was introduced as a factor transferred by DNA from a resistant strain, and permitting quantitative study of the number of cells transformed.[77] Recently, new transformations have included biochemically defined traits of sugar fermentation and oxidation.[78,79] It has been reported that virulence of crown-gall bacteria for plant hosts can be transmitted to avirulent strains by a DNA-containing preparation.[80] Table II shows a list of the transformations reported to be achieved with DNA preparations. Altogether about 21 capsular transformations are listed, involving at least 15 different polysaccharide antigens, and 3 transformations related to cell surface properties are recorded. In all, at least 30 biochemically distinct characters have been introduced *in vitro* by bacterial DNA into living cells of the homologous species. Other reports of transfers *in vitro* of drug sensitivity[91,92] or resistance[93,94] of antigenic[95] and enzymic[96] properties of bacteria induced by bacterial products, and of the transformation of a fibroma into a myxoma virus,[97,98] do not in general provide sufficient information to permit a conclusion about the nature of the change or the substances inducing them, or even in some cases whether the change was actually experimentally induced or spontaneous. Certain transformations by nuclease-sensitive agents and penicillin lysates[77] are certainly attributable to DNA, as are presumably certain protein-antigen[73, 87] and capsular-antigen[73, 81] shifts obtained in *in vivo* experiments with pneumococci. Another type of transformation—the transduction of *Salmonella*—which will be discussed in a later section, appears to be mediated by more complex agents.

[83] H. Ephrussi-Taylor, *Exptl. Cell Research* **2**, 589 (1951).
[84] S. Beiser and R. D. Hotchkiss, *Federation Proc.*, **13**, 486 (1954).
[85] G. Leidy, L. Hahn and H. E. Alexander, *J. Exptl. Med.* **97**, 467 (1953).
[86] R. Austrian, *J. Exptl. Med.* **98**, 35 (1953).
[87] R. Austrian and C. M. MacLeod, *J. Exptl. Med.* **89**, 451 (1949).
[88] R. D. Hotchkiss, *Proc. Natl. Acad. Sci. U. S.* **40**, 49 (1954).
[89] H. E. Alexander and G. Leidy, *J. Exptl. Med.* **97**, 17 (1953).
[90] H. Ephrussi-Taylor, *Exptl. Cell Research* **6**, 94 (1954).
[91] A. Voureka, *Lancet* **254**, 62 (1948).
[92] A. K. Saz and H. Eagle, *J. Bacteriol.* **66**, 347 (1953).
[93] O. Wyss, *Ann. N. Y. Acad. Sci.* **53**, 183 (1950).
[94] F. Roland and C. A. Stuart, *Antibiotics & Chemotherapy* **1**, 523 (1951).
[95] A. J. Weil and M. Binder, *Proc. Soc. Exptl. Biol. Med.* **66**, 349 (1947).
[96] M. U. Dianzani, *Experientia* **6**, 332 (1950); *Boll. ist. sieroterap. milan.* **29**, 161 (1950).
[97] G. P. Berry and H. M. Dedrick, *J. Bacteriol.* **31**, 50 (1936); **32**, 356 (1936).
[98] M. H. D. Smith, *Ann. N. Y. Acad. Sci.* **54**, 1141 (1952).

TABLE II

BACTERIAL TRANSFORMATIONS EFFECTED *In Vitro* WITH DNA PREPARATIONS

Character transferred	Species	Number of characters	Ref.
Capsular antigens			
Type III	*D. pneumoniae*	1	59
Types II, VI, XIV	*D. pneumoniae*	4	66
Types I, VIII	*D. pneumoniae*	2	73
Intermediate Type II	*D. pneumoniae*	1	81
Intermediate Type III	*D. pneumoniae*	2	82
Reconstituted Types III	*D. pneumoniae*	2 (to 4)	83
Deviant Types III	*D. pneumoniae*	1 (to 3)	84
Types *a*, *b*, *c*, *d*, *e*, *f*	*H. influenzae*	6	75
Mixed type *ab*	*H. influenzae*	1	85
Types I, II	*N. meningitidis*	1	76
Antigen S_1	*E. coli*	1	74
Other surface factors			
Rough (nonfilamentous) antigen	*D. pneumoniae*	1	82, 86
Extreme rough (filamentous)	*D. pneumoniae*	1	86, 100
M-protein antigen	*D. pneumoniae*	1 (to 2)	87
Drug resistances			
Penicillin	*D. pneumoniae*	3	77
Streptomycin	*D. pneumoniae*	2	77, 65
Streptomycin (high step)	*D. pneumoniae*	1	65, 88
Streptomycin (high step)	*H. influenzae*	1	89
Sulfanilamide	*D. pneumoniae*	1	79
Enzymic capacities			
Mannitol utilization	*D. pneumoniae*	1	79
Salicin	*D. pneumoniae*	1	78
Large colony (lactate?)	*D. pneumoniae*	1	90
Virulence in plant	*Agrobacterium*	1	80

e. Biological Nature of Transformation

Only a fraction of the cells present are permanently modified in a bacterial transformation. The fraction has been given as 0.2 to 5.0% in cultures of pneumococcus[88] and from 0.01 to 0.05% in *Hemophilus*.[75, 89] These yields are gradually being raised by improvements in the efficiency of the process so that yields of over 15% have recently been obtained with pneumococcus.[88] Qualitatively, there appears to be a sharply defined alteration in the properties of those cells which have been transformed. One may ask in what way this alteration is related to a mutation and to the normal genetic mechanisms of the bacteria.

(1) *Parallels between Genes and Transforming Agents.* If transforming

[99] R. D. Hotchkiss, *Exptl. Cell Research* Suppl. 2, 383 (1952).
[100] H. E. Taylor, *Compt. rend.* **228,** 1258 (1949).

factors are to be identified with bacterial genes, there should be unmistakable parallels between these entities. Genes of the higher organisms and bacterial transforming factors have been compared both from a structural or material point of view and functionally.[99] These biological entities both imply the material existence of specific substances at definite sites in the cell. Thus, just as many known genes can be assembled from known sources into one cell, so several independent transforming abilities can be introduced into the DNA of a single bacterial strain.[77, 79] On the other hand, like the alternative "mutant" forms of a given gene, the DNA factors for different antigenic and morphological traits[59,75,81,82,87] appear to exclude or even displace each other.[72,73,82,86,100] The exceptions to this last statement[81–83,101] are of a sort and frequency interpretable[101] in the same way as are exceptions among the gene series. Genes are, as we have seen, associated with DNA protein-containing chromosomes—structures having apparently grossly similar chemical composition along their length at different regions which can determine the most diverse biochemical properties of the cell. Likewise, the 30 different transformations mentioned in Table II are brought about by agents all found in the DNA fractions from the respective strains, fractions apparently uniform in chemical nature and composition. Each of these agents too, like the genes, is reduplicated whenever the cell which acquires it reproduces.

(2) *Mutations in Bacteria.* The functioning of DNA transforming agents, like that of genes, is principally studied by the observation of the mode of introduction of a new or variant property, called a mutation, from one strain into another which did not previously bear it.

In higher forms, a genic mutation is in part defined as a heritable change in the nuclear determinants which occurs as a discontinuous, discrete event and produces a corresponding change in the cellular properties (phenotype). If it is a nuclear determinant which mutates, the mutation should be inherited as a unit gene in crosses; mere transmissibility from mother to daughter cell does not distinguish between a nuclear mutation and a heritable change originating in the cytoplasm.

Obviously, in identifying bacterial mutations we are at some disadvantage. Objects resembling nuclei have been seen in bacteria,[102-105] but their chemical nature is not yet even outlined, and there is as yet only slight

[101] H. Ephrussi-Taylor, *Cold Spring Harbor Symposia Quant. Biol.* **16,** 445 (1951).
[102] G. Knaysi, "Elements of Bacterial Cytology," 2nd ed. Comstock Publishing Co., Ithaca, N. Y., 1951.
[103] C. F. Robinow, *Proc. Roy. Soc. (London)* **B130**, 299 (1942).
[104] R. Tulasne and R. Vendrely, *Compt. rend. soc. biol.* **141,** 674 (1947).
[105] K. A. Bisset, "*The Cytology and Life-history of Bacteria.*" E. and S. Livingstone, Ltd., Edinburgh, 1950.

evidence that they function cytologically[105-107] or genetically as sites of mutation[108] as do nuclei of the higher forms. Furthermore, since bacteria multiply by mother-daughter division, a simple hybridization technique is not available for testing the unit-particle transmissibility of a mutation. A somewhat analogous process, recombination in the K-12 strain of *E. coli*, has been used for this purpose in the very important work initiated by Lederberg,[109] but unfortunately the crossing between bacterial cells is a very rare event in these strains and has not yet been produced at all in most strains and species of bacteria. In any case, a host of discrete, discontinuous, heritable alterations in properties have been observed in bacteria and are now generally designated mutations. Among those in which the all-or-none spontaneous nature of the change has been demonstrated are virus resistance, drug resistance, and various biochemical deficiencies.

(3) *Transformations as Stepwise Transfer of a Mutation Pattern.* If we compare the manner in which bacterial mutant characters are acquired spontaneously, and that in which they are transferred by DNA, we find a number of parallels. First, when independent properties such as capsule synthesis and penicillin or streptomycin resistance are both being transferred from a single DNA preparation of a doubly marked strain, only one of these properties as a rule is acquired at a time by an individual transformed cell.[77,79,86] This and other examples appear to reflect the independent existence of different DNA particles determining different unit properties. These observations fit well with the fact that the bacterial strain furnishing the DNA has originally acquired these mutations separately in single steps and is presumed therefore to have unit genes corresponding to these very transforming units detected.

An analysis of the dose-response relation has indicated that the collision effective in transforming one cell in a pneumococcal microcolony requires only one particle of transforming agent.[110] Other recent data have shown that one single brief exposure to transforming agent can in certain instances introduce into an individual cell two factors if they both are present in the donor strain from which DNA was prepared.[79] This finding appears to indicate that certain pairs of transforming agents stand in special relation to each other, either chemically within one and the same DNA particle, or perhaps in respect to the way they can be accepted by a single cell.

A striking parallelism between the transforming factor and the hypo-

[106] K. A. Bisset, *Cold Spring Harbor Symposia Quant. Biol.* **16,** 373 (1951).
[107] B. Delaporte, *Advances in Genet.* **3,** 1 (1950).
[108] E. M. Witkin, *Cold Spring Harbor Symposia Quant. Biol.* **16,** 357 (1951).
[109] J. Lederberg, *Genetics* **32,** 505 (1947).
[110] A. W. Ravin, *Exptl. Cell Research* **7,** 58 (1954).

TABLE III

COMPARISON OF MUTATION PATTERNS IN SPONTANEOUS MUTANTS AND IN TRANSFORMATIONS EFFECTED BY THEIR DNA

Properties acquired spontaneously in donor strain	Properties acquired by strain transformed by donor DNA	Ref.
Capsule + penicillin resistance	Capsule, or resistance (1 step); very rarely, capsule + resistance	77
Capsule + streptomycin resistance	Capsule, or resistance (1 step)	77
Capsule + nonfilamentous	Capsule	59, 86
	Nonfilamentous	82, 86
	Rarely, capsule + nonfilamentous	86
Capsule + filamentous	Capsule	86
	Filamentous	86
Penicillin resistance (1 step)	Resistance (1 step)	77
High penicillin resistance, (multiple steps)	Resistance (1 step); then successive higher steps	77
High streptomycin resistance (multiple step type)	Resistance (1 step); then successive sive higher steps	65
High streptomycin resistance (single high step type)	Resistance (1 high step)	65, 88

thetical genes is found when the independent mutations being transferred are stepwise increases in drug resistance. Penicillin resistance is known to be acquired in stepwise spontaneous mutations in bacteria, each step permitting growth in a higher concentration of the drug.[111] When a highly resistant pneumococcal strain which has undergone multiple successive steps of mutation to resistance is the source of transforming agent, penicillin-sensitive cells are transformed only to the first, lowest level of resistance.[77] The product of the one-step process, treated with the same DNA from the multiply resistant strain, can now acquire the second step of resistance, and so on. In other words, transformation has transferred in stepwise fashion determinants which correspond exactly to determinants which had previously been successively acquired or produced in the spontaneous mutations of the donor strain. These findings are represented in Table III. The new resistant strains, like other transformed strains, can serve in turn as source of whatever DNA factors they have acquired through transformation. It is natural from these results to conclude that the DNA may itself be the entity which is the original site, as well as the transmissible form, of the mutation which occurred in the donor strain.

Streptomycin resistance also appears in quantitative mutational steps transferred stepwise in transformation. Here we have even more striking specificity of pattern, since, besides the stepwise resistance series, a still

[111] M. Demerec, *Proc. Natl. Acad. Sci. U. S.* **31**, 16 (1945).

more rare type of mutation gives in a single step a highly streptomycin-resistant mutant. If we have two strains, one highly resistant as the result of multiple cumulative mutations, the other as the result of the single high step, we can prepare two DNA transforming agents from two strains of different genetic makeup but outwardly similar properties. It is observed that each DNA preparation unmistakably reflects the history of its donor strain by transmitting the first level of resistance that strain had reached, which in one case is a low step, in the other a high step. Again we see that the extracted DNA represents not merely the attained resistance state of the donor bacteria, but actually appears to be a repository for the successive genetic mutational experiences of the donor strain.

(4) *Transforming Agents as Genes.* The bacterial DNA preparations, then, have the most important attributes of genes. It will be remembered that bacterial mutations and therefore bacterial genes had to be considered as less clearly defined than the nuclear genes and mutations of higher forms, chiefly because there is no sexual process generally available with which to test the unitary nature of their transmission. In the very process of transformation, however, we have this unitary transmission of hereditary determinants from cell to cell, in the form of DNA particles. Since this criterion is thereby satisfied, the bacterial genes and mutations can be taken to be analogous to those of higher forms, and at least some of the bacterial genes have been found to be in the cell DNA. There were already reasons for suspecting DNA to be important in the genetic mechanisms of the higher forms, and the results obtained with the bacteria indicate that the transforming agents are composed of DNA and act as if they were bacterial gene material separated from the parent cell.

There remains to be considered the proper role of the transforming agent in the cell from which it came. It must be pointed out again that mutant properties are merely the convenient markers by which the experimenter can follow a genetic process. Since each new mutation of bacteria appears to be reflected in the DNA components of the cells, there is every reason to suppose that a host of unidentified normal determinants are present in this same material. If not disturbed in the parent cell, the marked, and presumably also the unmarked, determinants will be repeatedly reduplicated and, in fact, represented in each of the potentially unlimited progeny of that cell. It is reasonable to conclude that the total array of these DNA determinants is the principal apparatus by which the mother cell passes on to its daughter cell its various inherited potentialities. The DNA particles, then, are not only to be considered as the site of certain mutations and source of certain transforming agents, but also as the fundamental cell components through which, in cell division as well as in fertilization, we suppose most or all properties are inherited in the different species.

3. Implications of a Genetic Role—Biochemical Specificity of DNA

Some 30 different transforming factors have been mentioned as occurring in DNA preparations of apparently similar properties from different bacterial strains. Indeed, it appears that as soon as new specific transforming agents are defined they are recognized in the DNA fraction of the appropriate cells. It must then be supposed that these preparations, highly purified according to the usual criteria, have latitude for specific differences of composition or configuration within the DNA structure itself, allowing for the many genetic factors observed and for the multitude of others to be expected.

Certainly some specific biological patterns are not inherited in the form of DNA or DNA-proteins. A number of plant viruses, at least, are PNA-proteins. The same may be true of cytoplasmic structural entities such as the mitochondria and microsomes, which have certain heritable aspects. Proteins, of course, can manifest and maintain specific patterns, and may well transmit them. Nevertheless, it is a striking fact that most living replicated units which have been investigated, including perhaps the majority of the viruses, are known to contain DNA, and those which do not, appear to contain its relative, PNA.

It may be well to recall here that such polysaccharides as glycogen, although sometimes described as "self-duplicating" or as templates capable of determining their own biosynthesis, are not such in reality. As Cori and Cori[112] have clearly indicated, "primers" of carbohydrate biosynthesis furnish appropriate end glycosidic residues upon which polysaccharide synthesis takes place in a pattern determined entirely by the enzymes present and not necessarily related to that of the "primer."

a. Specificity in Chemical Composition or Configuration

It has been described in Chapter 10 that DNA's of different species may show differences in purine and pyrimidine base content. There is, furthermore, considerable uniformity in the composition of the DNA isolated from different tissues of the same species. Once variations in base content were established, it became clear that DNA's might have a wide variety of different structures, as predicted from a consideration of the biological activities of the bacterial DNA's.

Indeed, new pyrimidine bases qualitatively distinct from the usual cytosine and thymine, have been found in a few sources. One of these, 5-methylcytosine, is apparently absent from many bacterial and virus DNA's[113-115] but constitutes an appreciable percentage of the total bases of

[112] G. T. Cori and C. F. Cori, *J. Biol. Chem.* **151,** 57 (1943).
[113] E. Vischer, S. Zamenhof, and E. Chargaff, *J. Biol. Chem.* **177,** 429 (1949).
[114] J. D. Smith and G. R. Wyatt, *Biochem. J.* **49,** 144 (1951).
[115] G. R. Wyatt, *J. Gen. Physiol.* **36,** 201 (1952).

calf thymus nucleic acid[116,117] and wheat germ DNA.[117] Certain of the T phages of *E. coli* are at present the only known natural sources of another cytosine base, 5-hydroxymethylcytosine.[118] These same viruses contain no ordinary cytosine,[119] whereas the aminopyrimidine of the host bacteria is exclusively cytosine.

Dramatic as their discovery may be, it is the very exclusiveness of these new bases which indicates that qualitatively different base composition is not the principal foundation for the finer details of DNA specificity. For there are to be explained not only interspecies differences but the existence of different DNA entities within each single species. Phage T_2, having only the hydroxymethyl form of cytosine, builds up a variety of specific DNA-determined factors (see below), as does also the T_7 virus (or Pneumococcus or *Hemophilus*) having only cytosine for an aminopyrimidine.

Similarly, the reported quantitative differences in the base compositions of the DNA from different species need have no connection with the genetic properties of this material. For it is the unfractionated DNA from each species (with the exceptions given below) in which heretofore these analytical differences have been shown. Since these are mixtures, presumably containing a great many different genetically specific entities, there is no indication whether or not these individual components are alike in their composition. What the analyses do show is that differences in the average base composition do occur between species and, therefore, different *arrangements* of bases must be possible in individual DNA molecules. It seems likely that different arrangements of bases in a nucleic acid chain—*with or without a difference in overall composition*—provide the means by which different specific molecules are constructed. At a still higher level of organization, there may be specific modes of bonding and folding of chains within the macromolecule which create unique structures. This might occur even when the arrangements of the bases along the chains do not greatly differ.

The most detailed physical structure proposed for DNA, that of Watson and Crick,[120] has features which not only approximately satisfy the requirements of the X-ray data,[121,122] but also suggest a basis for unique structures and their specific copying in genetic processes.[123] The structure is viewed as made up of double coaxial helices held to each other by hydrogen bonding between complementary pairs of purine and pyrimidine bases

[116] R. D. Hotchkiss, *J. Biol. Chem.* **175,** 315 (1948).
[117] G. R. Wyatt, *Biochem. J.* **48,** 581 (1951).
[118] G. R. Wyatt and S. S. Cohen, *Nature* **170,** 1072 (1952).
[119] A. Marshak, *Proc. Natl. Acad. Sci. U. S.* **37,** 299 (1951).
[120] J. D. Watson and F. H. C. Crick, *Nature* **171,** 737 (1953).
[121] M. H. F. Wilkins, A. R. Stokes, and H. R. Wilson, *Nature* **171,** 738 (1953).
[122] R. E. Franklin and R. G. Gosling, *Nature* **171,** 740 (1953).
[123] J. D. Watson and F. H. C. Crick, *Nature* **171,** 964 (1953).

(Chapter 13). Optimal bonding requires that adenine cross-link with thymine, and guanine with the cytosines, therefore insuring that one helix will of necessity be the complement of the other. One can imagine the double helix to be resynthesized upon a pattern furnished by either half. These hypothetical relations are in agreement with the generalization that the observed adenine-to-thymine and guanine-to-cytosine molar ratios of all DNA's are near unity although, taken the other way, adenine and guanine residues, for example, are often present in very different amounts.[124] This picture of DNA structure, then, in common with other less explicit ones, would attribute the fundamental specificities to variation in the sequences of the purine-pyrimidine pairs along the helical coils. As we have seen, the analytical data offer some support for this possibility, and yet offer no indications as to how many permutations in arrangement may actually occur.

b. Physical Evidence of Heterogeneity of DNA

Bacterial transformation, especially with multiply marked DNA, has indicated that different DNA's exist. Unfortunately, DNA does not prove to be antigenic, so its heterogeneity has not been confirmed immunologically.

Although most preparative methods tend to collect in one fraction all of the DNA of a general polymer size class, there are a few recent indications that not all DNA material behaves alike. Fractions differing in metabolic activity and salt solubility[125] and in binding to basic protein[126,127] have been recognized. Separations of a single species DNA on the latter principle have given fractions of somewhat different base composition.

Chemical analyses in the years since the bacterial transforming agents were recognized as DNA, have accordingly supported the view that DNA molecules, once thought to be alike and of simple construction, are chemically as well as biologically diverse, in accordance with the important genetic functions they have to fulfill.

II. Deoxypentose Nucleic Acids in Growth and Metabolism

Long before there was any convincing evidence that DNA's had a genetic role, a number of workers were interested in investigating their distribution and metabolism in tissues. Much of the findings can be summarized by saying that DNA molecules are metabolically relatively stable in most tissues and that they are produced in rather strict relation to growth. This

[124] E. Chargaff, *Experientia* **6,** 201 (1950); *Federation Proc.* **10,** 654 (1951).
[125] A. Bendich, *Exptl. Cell. Researeh* Suppl. 2, 181 (1952); A. Bendich, P. J. Russell, and G. B. Brown, *J. Biol. Chem.* **203,** 305 (1953).
[126] E. Chargaff, C. F. Crampton, and R. Lipshitz, *Nature* **172,** 289 (1953).
[127] G. L. Brown and M. Watson, *Nature* **172,** 339 (1953).

subject will be considered briefly, from the vantage point that has been attained by looking upon DNA's as genetic determinants.

1. Metabolic Stability of DNA

The facts already presented in Chapters 25 and 26 have indicated that atom groups from intermediary metabolites are not introduced as readily into tissue DNA's as they are into PNA's, proteins, and soluble cell constituents. This is true for the utilization of isotopically labeled nitrogen, carbon, and phosphorus compounds, and of intact purine and pyrimidine bases. It has been satisfactorily established by Hevesy, and others since, that an isotope is incorporated into DNA mainly when there is mitotic division, as in growing tissue, regenerating liver, or tumors. When once incorporated, the tagged atoms disappear from cellular DNA far slower than from PNA and other sites.

This metabolic stability is very likely an important requirement for materials which are to serve as pattern-makers for inheritance. Every growing organism has to build up DNA, but, when not actively growing, it is more likely to keep its biological heritage intact if it can minimize the participation of that DNA in the wasteful equilibria which involve the breaking down and rebuilding of so many cellular constituents.

As pattern-making components, DNA-containing structures have to be duplicated before the cell can divide. It is interesting in this connection that the newly incorporated DNA-phosphorus in growing liver and intestinal mucosa[128] (and also lung in a later work[129]) has been reported to be just about sufficient to supply new DNA-P to both daughter cells from each mitosis. In this work, the amount of isotopic phosphorus incorporated into the tissue DNA after an injection of labeled phosphate was related to the mitotic activity of the tissue. It is implied that the preexisting DNA-phosphorus, and probably the DNA itself, is not retained but is entirely renewed during a mitosis. This most interesting inference should not be accepted literally at present, since the calculation is fraught with many hazards and may be unreliable. It depends upon the accuracy of assay of the number of mitoses, upon the assumption that the analytical changes in DNA-P are directly and exclusively referable only to these dividing cells, and upon the assumption of the nature and isotope level of a changing precursor pool; and it is very critically dependent upon the purity of the assayed DNA fraction. Even if correct, the results may merely indicate that other cells, besides the ones seen in mitosis, are duplicating their DNA. It was not feasible in the present instance to demonstrate the destruction or elimination of preexisting DNA; in most studies of this sub-

[128] C. E. Stevens, R. Daoust, and C. P. Leblond, *J. Biol. Chem.* **202,** 177 (1953).
[129] C. E. Stevens, R. Daoust, and C. P. Leblond, *Can. J. Med. Sci.* **31,** 263 (1953).

ject, it is characteristically found that, once labeled, tissue DNA retains its phosphorus and other markers through long periods of growth. In general, it may be said that cell DNA goes through changes primarily only in connection with cellular division processes, and then principally only augmentation.

a. DNA Content of Tissues

Analytical studies at the tissue, rather than at the cellular, level have provided a great number of measurements relating the DNA content to the dry or wet weight of various tissues under different physiological, nutritional, or metabolic conditions. If we choose to accept the average DNA content per nucleus, or per cell, as constant (as discussed above and in Chapters 16 and 19) at all times in a given tissue, then the DNA analysis per weight of tissue can be taken as an inverse measure of the way cell weights are changing under the experimental conditions being studied.

Other factors, such as replication of chromosomes or of chromosome strands, and differences in ploidy, would tend to complicate this picture somewhat. The cell DNA content increases in cyclical fashion from a basal amount to the double amount and back as the cell goes through a whole division cycle. For mass analyses of adult animal tissues, the average over this period does not represent a large source of variation, since divisions are relatively rare; it may be the explanation for the reported higher DNA content of tumor tissues[130,131] and embryonic tissues of sheep,[132] mouse,[133] grasshopper,[134] and sea urchin.[135] In general, it appears that the DNA content represents the least variable parameter of a tissue—so that it is in fact reasonably correct to use analytical changes of percentage DNA content as expressions of the changing average mass of the tissue cells. Thus, the apparently higher DNA content of livers of female rather than of male rats turns out to signify the smaller average mass of the female rat liver cell, the total DNA per cell and per animal being the same for both sexes.[131] The equivalent is true for apparent decrease of DNA during pregnancy, and for the increase of DNA found after fasting or the feeding of various deficient diets.

Similarly, when another parameter is expressed as a ratio to DNA, e.g. PNA-phosphorus to DNA-phosphorus, the ratio may be a fairly direct

[130] W. C. Schneider, *Cancer Research* **5,** 717 (1945).
[131] R. Y. Thomson, F. C. Heagy, W. C. Hutchison, and J. N. Davidson, *Biochem. J.* **53,** 460 (1953).
[132] J. N. Davidson and C. Waymouth, *Biochem. J.* **38,** 39 (1944).
[133] M. Alfert, *J. Cellular Comp. Physiol.* **36,** 381 (1950).
[134] H. Swift, *Intern. Rev. Cytol.* **2,** 1 (1953).
[135] R. D. McMaster, Thesis, Zoology Dept., Columbia University, New York, 1952. From L. C. Sze, *J. Exptl. Zool.* **122,** 577 (1953).

measure of the PNA per cell. Within limitations imposed by questions of ploidy, etc., one may expect a comparison of DNA contents between different tissues of the same species to provide an indirect measure of the relation between the average cell masses of these tissues.

These questions have been discussed in some detail in Chapter 16

b. DNA as a Measure of Growth

If the percentage DNA content of a tissue gives information mainly about such parameters as cell mass, the *total amount of DNA* present is an important measure of cell number and reflects the occurrence of growth. Among the systems which have been followed in this way are: the development of fibroblasts,[136] leucocytes,[137,138] various organ tissues,[139] and viruses[140] in tissue cultures, and of malaria parasites in erythrocytes.[141] The total DNA per liver serves as a measure of liver regeneration; during the rapid phase of growth shortly after hepatectomy, the DNA per nucleus becomes higher than normal, so that for a time the DNA is increasing faster than cell number, subsequently stopping at the point when the amount of DNA originally present in the total liver has been restored.

The usefulness of nucleic acid phosphorus (especially the DNA part) as a measure of growth was recognized empirically,[142-144] and then received logical support when the constancy of DNA in the nucleus was discovered. As has been pointed out in Chapter 16, it seems likely that DNA will come to serve more and more either as a measure of cell number or, as a corollary to this, as a basis of reference to which other analyzed components are expressed in the form of an analytical ratio.

There is some evidence that DNA duplication at the cellular level occurs well in advance of cell division. Photometric measurements of individual animal and plant nuclei have indicated that new DNA synthesis occurs early in interphase of mitotic or meiotic cycles,[145] or between interphase and early prophase.[12,146-148] Chemical analyses related to changes in mitotic

[136] J. N. Davidson and C. Waymouth, *Biochem. J.* **37,** 271 (1943).
[137] E. E. Osgood, J. G. Li, H. Tivey, M. L. Duerst, and A. J. Seaman, *Science* **114,** 95 (1951).
[138] E. E. Osgood, H. Tivey, K. B. Davison, A. J. Seaman, and J. G. Li, *Cancer* **5,** 331 (1952).
[139] H. W. Gerarde, M. Jones, and T. Winnick, *J. Biol. Chem.* **196,** 69 (1952).
[140] L. T. Atlas and G. A. Hottle, *Science* **108,** 743 (1948).
[141] P. R. Whitfeld, *Nature* **169,** 751 (1952); *Australian J. Biol. Sci.* **6,** 234 (1953).
[142] I. Berenblum, E. Chain and N. G. Heatley, *Biochem. J.* **33,** 68 (1939).
[143] E. N. Willmer, *J. Exptl. Biol.* **18,** 237 (1942).
[144] J. N. Davidson, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 50 (1947).
[145] J. Pasteels and L. Lison, *Arch. biol. (Liége)* **61,** 445 (1950).
[146] H. Ris, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 158 (1947).
[147] H. Swift, *Proc. Natl. Acad. Sci. U. S.* **36,** 643 (1950).
[148] J. H. D. Bryan, *Chromosoma* **4,** 369 (1951).

frequency,[149] or to stages in synchronously dividing plant tissue,[150,151] and radioautographic estimation of DNA-phosphorus incorporation,[152] have led to similar conclusions (Chapter 19). These results suggest that new DNA for the daughter nuclei is available before division begins.

2. The Significant Role of DNA in Bacteriophage

Growth of living matter involves, as we have seen, the duplication of DNA-containing structures, and is in all probability genetically oriented by these entities. One may expect that the growth of intracellular viruses will, among other things, require the production of new specific DNA's within a cell already organized for the production of the host's own DNA determinants. In the infections of *E. coli* with the T bacteriophages, the study of these relationships has to some degree confirmed and extended the conceptions already presented in this chapter.

a. Composition of the T Phages

The so-called T coliphages lytic for the host bacteria *E. coli* are small, hexagonal or rounded, tail-bearing objects principally made up of protein (around 60%) and DNA (about 40%), together with small amounts of lipid. Although these viruses were once thought to be nucleoproteins, it is now considered that the DNA is mechanically, rather than chemically, confined by the protein.

Intact phage particles are agglutinated by antiserum which reacts with the protein, but are not affected by deoxyribonuclease. After plasmolysis, however, by rapidly diluting phage suspensions from high into low salt concentrations,[153] the DNA of T_2, T_4, and T_6 partly passes into solution[154] and all of it can now be rendered soluble by deoxyribonuclease.[154] The remaining sedimentable "ghosts" appear to be collapsed and ruptured phage membranes[153] and are still precipitable by the antiphage serum.[155] If phages are labeled with isotopic phosphorus (P^{32}) or sulfur (S^{35}), the latter is all found in the protein ghosts and the P^{32} is all found in the DNA made hydrolyzable by the rupture of the protein membranes.[155]

Apparently, then, phage protein exists largely as a membrane surround-

[149] J. M. Price and A. K. Laird, *Cancer Research* **10,** 650 (1950).
[150] M. Ogur, R. O. Erickson, G. U. Rosen, K. B. Sax, and C. Holden, *Exptl. Cell Research* **2,** 73 (1951).
[151] A. H. Sparrow, M. J. Moses, and R. J. Dubow, *Exptl. Cell Research* Suppl. 2, 245 (1952).
[152] S. R. Pelc and A. Howard, *Exptl. Cell Research* Suppl. 2, 269 (1952).
[153] T. F. Anderson, *Botan. Rev.* **15,** 464 (1949).
[154] R. M. Herriott, *J. Bacteriol.* **61,** 752 (1951).
[155] A. D. Hershey and M. Chase, *J. Gen. Physiol.* **36,** 39 (1952).

ing a mass of free or loosely bound DNA. This is not to be taken as a complete description of T phage structure, and by no means as a picture necessarily applicable to other viruses. However, it does make possible a differentiation of the functions of the protein and DNA components.

b. Life Cycle of Virulent Phage—Role of the Protein and DNA

When coliphages are added to *E. coli* cells in a suitable environment, the phage particles are rapidly adsorbed in random distribution upon the bacteria. They adhere to the cells through their "tails," and antisera which react with the tail protein can, if added in advance, prevent attachment. Adsorption soon becomes irreversible, and the bacterium is now killed, i.e., no longer able to produce more bacteria, becoming instead a producer of phage. After a latent period, which in part is determined by the growth medium and the temperature, the cell lyses and liberates a large number of new phage particles. These phenomena are indicated schematically in Fig. 2.

The surface of the phage (probably the tail) is the part which makes contact with the bacterial cell, and it is in fact possible to obtain an abortive "infection" with the protein ghosts alone.[154] If no DNA is present, the ghost kills the cell and no phage is formed. This finding suggested that DNA was directly or indirectly required for new phage production. It turns out that DNA plays a major role as soon as infection has been established.

Hershey and Chase have made a classic demonstration of the different roles of DNA and protein of both damaged and intact phage.[155] Taking advantage of isotopic marking as already mentioned, these investigators clearly showed that the DNA (P^{32}) of infecting T_2 or T_6 phages enters into the infected cell, whereas the protein (S^{35}) does not. When infected cell suspensions were subjected to the vigorous shearing force of a mechanical blendor, phage protein was stripped off, leaving an infected cell containing all the phage DNA and only a small part (never over 20%) of the protein. Even that part of the protein which remained attached made no isotopic contribution to the new phage that was synthesized, but could be recovered completely among the bacterial debris after premature or ultimate normal lysis. Since the stripped cells still produced the normal amount of virus progeny, it appeared that phage DNA by itself is capable of carrying forward the process of infection, and that the protein is no longer needed after the DNA has been introduced into a host cell. Phage DNA has, however, never been successfully used in separated form to initiate infection. The impression has grown that phage protein is a protective membrane shielding phage DNA from deoxyribonuclease and other influences and serving to insure its introduction into the cell. It has been suggested that

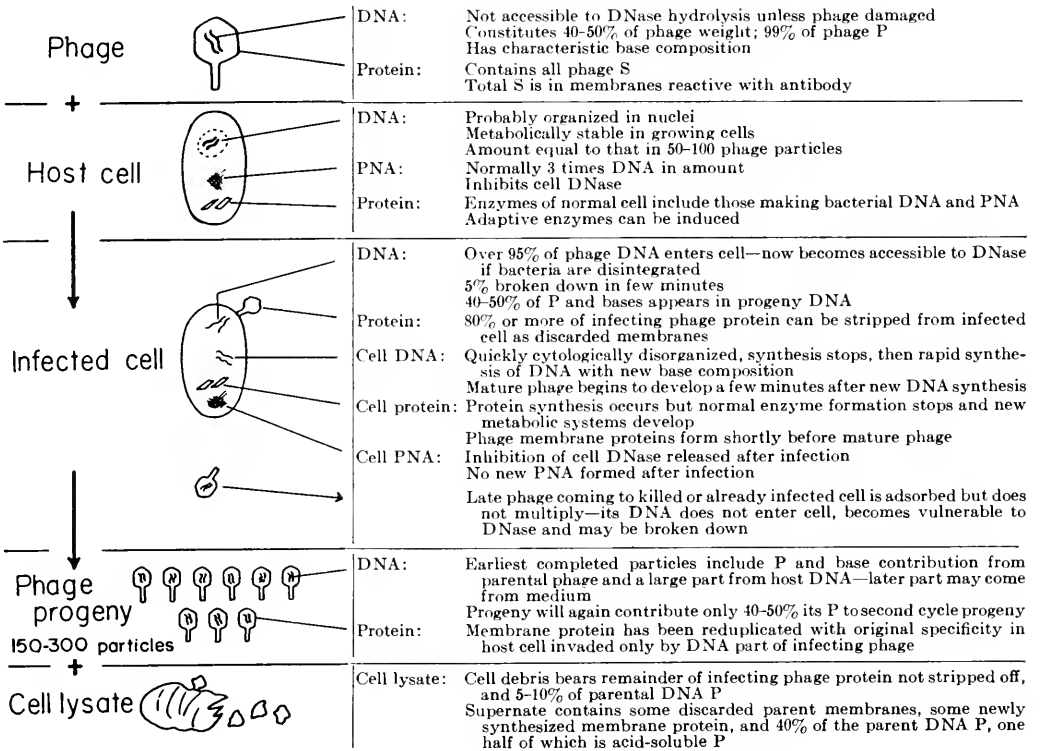


FIG. 2. Process of infection of *E. coli* with T phages.

the DNA may even be "injected" into the host cell through the phage tail structure.

Other observations made with labeled phage support this general picture. In all cases, protein, recognized by its S^{35} content (and sometimes by its amino acid composition), is the material which combines with specific antiserum or with the surface of susceptible normal or with heat-killed or disintegrated bacteria and becomes easily sedimentable. These properties are characteristic of the protein fraction whether it is in intact phage, in osmotically produced ghosts, in the material stripped from infected cells, or in the material not stripped off but recovered later with the bacterial debris. The DNA, on the other hand, behaves differently at different sites. It is not attacked by deoxyribonuclease either in intact phage or during or after it is transferred to the infected bacterium. Only in a system disturbed by some intervention which also disrupts phage reproduction has the DNA been made vulnerable to nuclease. Before infection, damage to phage membranes will expose DNA; after infection disintegration of bacterial membranes will suffice.[155,156] When adsorbed on bacteria previously heat-killed, frozen and thawed, or otherwise disintegrated, the protein of normal phage is bound, but the DNA becomes nuclease-vulnerable and can be readily extracted. It may have been extruded or injected upon, instead of into, the damaged cells. A somewhat similar accident befalls the DNA of certain T phages added to already infected cells. In such a case, the superinfecting phage begins to disintegrate spontaneously, giving off P-containing fragments,[157] and fails to introduce its phage characteristics into the progeny,[158] although the DNA may not become markedly susceptible to external deoxyribonuclease. With certain other T phages, there is little breakdown of late-coming phage but its phosphorus still is not taken into new phage.[159]

On the other hand, when it is the phage which is biologically inactivated (by formaldehyde), adsorption on the cells does not result in release of DNA either into the cell or in a state susceptible to nuclease from without.[155] Infection is not established and, in this case, mechanical stripping of the cell-phage complex removes DNA along with the phage coats.

Intact T phage, then, displays all the properties of its protein but not those of its DNA; after physical damage both components are revealed. Phage DNA is therefore considered to be free or very loosely combined inside a protective membrane comprised of the phage protein. At infection the DNA passes into the inside of the bacterial cell; the protein remains at

[156] A. Siegel, S. J. Singer, and S. G. Wildman, *Arch. Biochem. and Biophys.* **41,** 278 (1952).

[157] S. M. Lesley, R. C. French, A. F. Graham, and C. E. van Rooyen, *Can. J. Med. Sci.* **29,** 128 (1951).

[158] R. Dulbecco, *J. Bacteriol.* **63,** 209 (1952).

[159] A. F. Graham, *Ann. inst. Pasteur* **84,** 90 (1953).

the surface and probably plays no role after infection is established. Whatever happens after this appears to be determined by the DNA portion.

c. Genetic Aspects of Phage Reproduction

In the subsequent stages of T phage reproduction, quantities of phage protein membranes and phage DNA are synthesized in the infected cell. These are specific macromolecular materials different from any of the components of the uninfected host. It is generally assumed that such species-specific materials are produced under the influence of specific genetic determinants (even when these have not yet been recognized in mutant forms suitable for direct genetic experimentation). Accordingly, the biochemical processes leading to the production of these constituents were presumably initiated through the genetic action of that part of phage which is transferred to and retained by the infected host—the significant part identified from its phosphorus content as DNA.

The text of Fig. 2 gives a summary of the chemical processes observed during infection of *E. coli* with T phages. A few of the findings given there have only been observed with T_2 (or T_6) phage—it is likely that all are essentially true for the "T-even" (2, 4, and 6) phages and their various mutants and, so far as is known, probably for the other T phages as well. References not otherwise covered include those to: chemical analyses of host cells[160] and of phage;[118,160-162] study of infected cells as to changes of cell organization,[163] metabolism,[164-167] or protein synthesis;[160,168] degradation and utilization of input phage;[155,157,159,169,170] recognition of synthesized phage,[171] its DNA,[156,172] its protein,[173-175] or the origin of its components.[165,176-182]

[160] S. S. Cohen, *J. Biol. Chem.* **174,** 281 (1948).
[161] A. R. Taylor, *J. Biol. Chem.* **165,** 271 (1946).
[162] R. M. Herriott and J. L. Barlow, *J. Gen. Physiol.* **36,** 17 (1952).
[163] S. E. Luria and M. L. Human, *J. Bacteriol.* **59,** 551 (1950).
[164] S. S. Cohen, *Cold Spring Harbor Symposia Quant. Biol.* **18,** 221 (1953).
[165] L. M. Kozloff, *Cold Spring Harbor Symposia Quant. Biol.* **18,** 209 (1953).
[166] A. B. Pardee and R. E. Kunkee, *J. Biol. Chem.* **199,** 9 (1952).
[167] A. D. Hershey, J. Dixon, and M. Chase, *J. Gen. Physiol.* **36,** 777 (1953).
[168] L. Siminovitch, *Ann. inst. Pasteur* **84,** 265 (1953).
[169] L. M. Kozloff, *J. Biol. Chem.* **194,** 95 (1952).
[170] J. D. Watson and O. Maaløe, *Biochim. et Biophys. Acta* **10,** 432 (1953).
[171] A. H. Doermann, *J. Gen. Physiol.* **35,** 645 (1952).
[172] A. D. Hershey, *J. Gen. Physiol.* **37,** 1 (1953).
[173] C. Levinthal and H. Fisher, *Biochim. et Biophys. Acta* **9,** 419 (1952).
[174] C. Levinthal and H. Fisher, *Cold Spring Harbor Symposia Quant. Biol.* **18,** 29 (1953).
[175] F. Lanni and Y. T. Lanni, *Cold Spring Harbor Symposia Quant. Biol.* **18,** 159 (1953).
[176] L. L. Weed and S. S. Cohen, *J. Biol. Chem.* **192,** 693 (1951).

Biochemical studies indicate that host cell metabolism after infection is almost exclusively diverted to phage production (Chapter 26). Following infection, total protein synthesis goes on, and after a few minutes delay there is active DNA synthesis, but normal host enzymes or host DNA are apparently no longer being formed. The respiration and metabolism, which but for the infection would have been increasing in rate, remain more or less constant while the phage multiplies several hundredfold. This in itself suggests that the enzymes whose action is being observed are those of the no longer growing host cell, and not enzymes attributable to the phage. Not only do the host's respiratory and adaptive enzymes fail to increase after infection, but the cell PNA does not increase or undergo metabolic exchange. It has been suggested that intermediates for PNA synthesis are shunted toward DNA synthesis,[183] but there is little direct evidence to support this view; furthermore, certain enzymes which may be involved in ribose phosphate formation are still active after the infection.[184]

Clearly defined shifts in the synthesis of pyrimidine bases, however, occur under the influence of phage DNA. The already mentioned 5-hydroxymethylcytosine, never observed in the uninfected host, begins to be synthesized soon after infection with the T-even phages, which contain this base.[164,172] Also, a strain of *E. coli* which requires preformed thymine for growth begins rapidly to produce this base when infected with phage.[164] These may be looked upon as evidences that phage DNA exerts a profound organizing effect, as well as what might be merely adaptive shifts, in the metabolism of the bacterial cell which it infects.

Certain phage properties have been observed in mutant forms and have been followed through genetic processes. These are chiefly properties relating to the adsorbability on different bacterial strains or to the mechanism of lysis and consequently to plaque formation). Except that they may further mutate, these traits are reproduced indefinitely in a pure phage line, presumably being carried on through the specific DNA. New combinations, however, are produced when suitable dissimilar, but related, phages simultaneously infect the same cell.[185-187] Since these new combinations of prop-

[177] L. M. Kozloff, K. Knowlton, F. W. Putnam, and E. A. Evans, Jr., *J. Biol. Chem.* **188,** 101 (1951).

[178] A. L. Koch, F. W. Putnam, and E. A. Evans, Jr., *J. Biol. Chem.* **197,** 105 (1952).

[179] F. W. Putnam, D. Miller, L. Palm, and E. A. Evans, Jr., *J. Biol. Chem.* **199,** 177 (1952).

[180] L. W. Labaw, *J. Bacteriol.* **62,** 169 (1951).

[181] G. S. Stent and O. Maaløe, *Biochim. et Biophys. Acta* **10,** 55 (1953).

[182] O. Maaløe and N. Symonds, *J. Bacteriol.* **65,** 177 (1953).

[183] S. S. Cohen, *Nature* **168,** 746 (1951).

[184] S. S. Cohen and L. Roth, *J. Bacteriol.* **65,** 490 (1953).

[185] M. Delbrück and W. T. Bailey, Jr., *Cold Spring Harbor Symposia Quant. Biol.* **11,** (1946).

erties are in turn transmissible along with the new phage DNA, it is inferred that the original DNA determinants in some way have recombined to produce new determinants, just as they may do in bacterial transformations. It has been concluded from intricate analysis of this process that the recombination must occur between nearly completed phage DNA's, since recombinants usually are not replicated in the original host cell and do not occur after maturation.[188]

A somewhat different kind of recombination in a mixedly infected cell may result in the incorporation of one type of DNA within a protein membrane corresponding to another type. This "illegitimate" phage bears for one generation cycle only the host specificity of the membranes which it has acquired, then reestablishes in the next infection cycle the normal membrane and host specificity presumably corresponding to the DNA which it had borne throughout this time.[189,190]

Thus, although the detailed biochemical processes are by no means understood, it is clear that the DNA of an infecting T phage commandeers the nucleic acid and protein metabolism of the host cell much as though it were a genetic entity dominant over the host genes. The specificity of the new syntheses induced is predetermined by the infecting DNA and further modification appears to occur only when, presumably, the DNA itself has been modified in some manner.

d. Origin and Fate of Phage DNA

The various biological species have, of course, had to evolve pathways of synthesis for the unique DNA's which they bear, and, in general, it seems to be true that precursors at different levels of complexity may be taken from the environment to build up this all-important material. Coliphages first tend to utilize host DNA but when this is used up, nucleosides purine bases, and smaller components serve as sources of phage DNA.[165, 176,181,191] The relative quantities used depend upon the requirement, i.e., upon the DNA content of the phage produced,[180] and upon the number of particles formed per cell at the time of assay. Hershey has shown that a relatively constant amount of phosphorus and specific pyrimidine bases

[186] A. D. Hershey and R. Rotman, *Genetics* **34,** 44 (1949).

[187] S. E. Luria and R. Dulbecco, *Genetics* **34,** 93 (1949).

[188] N. Visconti and M. Delbrück, *Genetics* **38,** 5 (1953).

[189] A. D. Hershey, C. Roesel, M. Chase, and S. Forman, *Carnegie Inst. Wash. Yearbook* **50,** 195 (1951).

[190] A. Novick and L. Szilard, *Science* **113,** 34 (1951).

[191] Of course, higher animals decompose and utilize fragments of ingested DNA in their digestive systems, but Marshak and Walker[192] report that intravenously injected chromatin itself is a relatively efficacious source of labeled phosphorus for liver nuclei.

[192] A. Marshak and A. C. Walker, *Am. J. Physiol.* **143,** 235 (1945).

accumulate in a precursor pool from which phage is steadily produced after a few minutes of the latent period have elapsed.[172] Some of the more general findings are indicated in Fig. 2.

One of the important questions concerning precursors for phage DNA appears to be how the parental nucleic acid is utilized. There is good evidence from several sources that half or somewhat more of the parental phage P^{32} [155,157,169] and purine carbon[170] is discarded in low-molecular debris[157] at each cycle of infection.[189,193] There does not appear to be, therefore, a uniquely retained stable portion, and there is no indication whether the "discarded" fragments come from a genetically unessential portion or from determinants themselves, which may be degraded as a result of their functioning. In bacteria[194] and, as already mentioned, in animal tissues, the enhanced metabolic exchange of DNA at the time of cell division does not usually appear to bring about the loss of the phosphorus or other markers already incorporated.

e. Symbiotic Phages and Other Viruses

Most phages have not yet been sufficiently well prepared and analyzed to indicate whether or not they infect their hosts with DNA as do the T phages. It is, however, known that some of them contain DNA. This is true of certain staphylococcal phages, and the control of the DNA synthesis in infected staphylococci appears to be decisive for phage multiplication.[195]

One group of phages which have recently been much studied are the symbiotic or "temperate" phages which may be carried for many generations in an infected bacterial host, and only from time to time spontaneously lyse an occasional host cell to liberate free virus. Such phages are relatively difficult to prepare in pure form, and little is as yet known about their chemical nature, although DNA has been found associated with some of them.

When these temperate viruses do lyse their host, there are some signs of involvement of DNA. It is possible to induce lysis by some of these carried phages when the host culture is irradiated with ultraviolet or treated with certain mutagenic agents.[196] This observation itself has occasioned the inference that the "provirus" precursor was associated with the DNA and the genetic equipment of the host cell. Of interest in this connection is the fact that the symbiotic virus, λ, of *E. coli* K-12 is transmitted in sexual recombination processes in some form of linkage with the entities responsible for the genetic marker, galactose IV.[197]

[193] O. Maaløe and J. D. Watson, *Proc. Natl. Acad. Sci. U. S.* **37,** 507 (1951).
[194] L. W. Labaw, V. M. Mosley, and R. W. G. Wyckoff, *J. Bacteriol.* **59,** 251 (1950).
[195] W. H. Price, *J. Gen. Physiol.* **33,** 17 (1949).
[196] A. Lwoff, *Ann. inst. Pasteur* **84,** 225 (1953).
[197] E. Lederberg and J. Lederberg, *Genetics* **38,** 51 (1953).

Once lysis has been initiated by, for example, ultraviolet irradiation of the host cells, net DNA synthesis is temporarily blocked, then later resumed at an increased rate,[168] during the time that the temperate phage is rapidly accumulating in the cell prior to lysis. This characteristic effect is similar to that in cells synthesizing the virulent phages and indicates that both kinds of phage production are accompanied by profound disturbance of cell DNA metabolism. In contrast, however, the optical density increases, and PNA and enzyme synthesis continue to occur almost uninterruptedly up to the time of lysis in ultraviolet-induced temperate systems.[168] They are blocked in typical virulent phage systems as already described.

Other viruses which have been said to contain DNA are psittacosis,[198,199] vaccinia,[200] rabbit papilloma,[201] swine influenza,[202] and influenza PR8,[203] the last having a low content variously reported as from 0.3 to 2% DNA.[202, 204-206] DNA has also been found in the inclusion bodies of rabies,[207] and neurovaccinia,[207] molluscum contagiosum,[207,208] verruca (warts),[207,209] in various insect viruses,[115] and in *Rickettsiae*.[210,211] It should not be forgotten, however, that some plant viruses and perhaps a few animal viruses contain PNA and apparently no DNA.

f. Transduction—A Transformation Mediated by Phage

One of the remarkable processes brought about by certain symbiotic or temperate phages, called transduction, is so analogous to transformation by certain bacterial DNA's that it should briefly be mentioned here, although there is as yet no demonstration that DNA is specifically involved.

There were elicited from *Salmonella typhimurium* cells particulate preparations able to transfer heritable traits to other strains of *Salmonella*.[212]

198 P. Lépine and V. Sautter, *Ann. inst. Pasteur* **72,** 174 (1946).
199 H. R. Morgan, *J. Exptl. Med.* **95,** 277 (1952).
200 C. L. Hoagland, G. I. Lavin, J. E. Smadel, and T. M. Rivers, *J. Exptl. Med.* **72,** 139 (1940).
201 A. R. Taylor, D. Beard, D. G. Sharp, and J. W. Beard, *J. Infectious Diseases* **71,** 110 (1942).
202 A. R. Taylor, *J. Biol. Chem.* **153,** 675 (1944).
203 A. R. Taylor, D. G. Sharp, D. Beard, J. W. Beard, J. H. Dingle, and A. E. Feller, *J. Immunol.* **47,** 261 (1943).
204 C. A. Knight, *J. Exptl. Med.* **85,** 99 (1947).
205 C. A. Knight, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 115 (1947).
206 A. F. Graham, *Can. J. Research* **E28,** 186 (1950).
207 H. Hydén, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 104 (1947).
208 J. L. Melnick, H. Bunting, W. G. Banfield, M. J. Strauss, and W. H. Gaylord, *Ann. N. Y. Acad. Sci.* **54,** 1214 (1952).
209 H. Blank, M. Buerk, and F. Weidman, *J. Invest. Dermatol.* **16,** 19 (1951).
210 J. D. Smith and M. G. P. Stoker, *Brit. J. Exptl. Pathol.* **32,** 433 (1951).
211 G. R. Wyatt and S. S. Cohen, *Nature* **170,** 846 (1952).
212 N. D. Zinder and J. Lederberg, *J. Bacteriol.* **64,** 679 (1952).

A number of characters were transferred, including nutritional, fermentative, drug-resistance, and antigenic traits. Only one new property was observed to be transferred at a time to an individual cell, however, a feature by which transduction appears more closely related to transformation than to sexual recombination in microorganisms. The sedimentability of the agent and its stability to deoxyribonuclease, and the relatively low frequencies of transduction, at present distinguish the process from transformation. The active agent has now been traced to the phage particles liberated upon lysis of the donor strain.[213] The lysis may either be the spontaneous lysis by a symbiotically carried phage or that occasioned by introduction of the same temperate phage into a donor strain upon which it has a lytic action. The occasional effective phage particle appears to have conveyed a genic factor from the donor strain (its previous host) and introduced it (not necessarily establishing phage infection at the same time) into the new strain. Analogous transfers of two characters in *S. typhosa* by a virulent phage have been reported.[214]

III. Other Aspects of DNA Function

Although one would be unjustified from present knowledge either to assert or to deny that nuclear gene material of all species is composed of DNA, one should certainly not be misled into assuming that all DNA has a specific genetic role. We have seen that there is little reason to hold, for example, that all of a pneumococcus-transforming DNA preparation is made up of specific genetic determinants, whether identified or unidentified. It may very likely be as unjustified as assuming all proteins to be enzymes. It has often been suggested that some of the chromatin DNA of chromosomes has a function different from that of the rest (heterochromatin and euchromatin[215]). So, too, there have been indications that a certain part of the DNA of bacteriophages could undergo ultraviolet irradiation[216,217] or suffer atomic disintegration[218] without effect upon phage proliferation. These considerations lead us again to recall the fact that parental phage DNA-phosphorus and DNA-adenine are recovered only incompletely (35 to perhaps 50%) in the phage progeny. The conserved portion is not passed on intact, but is distributed, at least somewhat diluted, among the progeny, and it is not uniquely conserved, but again diluted, and partially

[213] N. D. Zinder, *Cold Spring Harbor Symposia Quant. Biol.* **18,** 261 (1953).
[214] L. S. Baron, S. B. Formal, and W. Spilman, *Proc. Soc. Exptl. Biol. Med.* **83,** 292 (1953).
[215] J. Schultz, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 179 (1947).
[216] S. Benzer, *J. Bacteriol.* **63,** 59 (1952).
[217] R. Dulbecco, *J. Bacteriol.* **63,** 199 (1952).
[218] A. D. Hershey, M. D. Kamen, J. W. Kennedy, and H. Gest, *J. Gen. Physiol.* **34,** 305 (1951).

dissipated in a second infection cycle. This might suggest that the genetically "functional" portion of the DNA is actually the half or more, the isotope of which is liberated as low-molecular substances during the process of reduplication. Once making this assumption, one might go so far as to suppose that all of the parent DNA is broken down in exerting its genetic functions, and a portion of the isotope only is reabsorbed from the metabolite pool. Although the studies with mammalian tissue already cited suggest that mitotic division also involves a wasteful synthesis of an extra duplicate portion of DNA, for the reasons already given this conclusion is not safely established.

Also more or less in opposition to these last views is the finding that carbon, nitrogen, and phosphorus of DNA are, in general, extraordinarily stable toward exchange, even during growth when incorporation of isotopically marked atoms is most rapid. Furthermore, the cytochemical studies of nuclei, described above and in Chapter 19, have led to the inference that the regularities in DNA content from cell to cell are a direct consequence of the regularities with which genetic material is distributed through known processes, none of which requires or suggests a breakdown. It does not, therefore, seem probable that DNA is generally broken down, in whole or in part, either during cell division or in performing its genetic functions. Neither do we find, on those occasions when a part of it is broken down, that this degradation has an obvious relation to genetic functioning, or that the one part of the DNA is in any obvious way less "essential" than the other.

1. Other than Genetic Roles for DNA

Nevertheless, the distribution of DNA in cells and tissues does not always seem to fit an exclusively genetic role. It is true, as already described, that the irregularities as well as the regularities in nuclear DNA distribution can generally be correlated with the sometimes irregular replications or distributions of the genetic complement. There is sufficient variability in the DNA of individual tissue nuclei, however, to raise the questions whether the rather striking fixed levels of DNA content reported actually represent the aggregate diploid set of genes, or chromosomes bearing nongene DNA also, and, if so, whether before or after duplication in preparation for cell division; or if they are merely modal values of a statistical distribution[15] of different degrees of replication. Indeed, it has been calculated that a *Drosophila* cell contains enough DNA for many replicates of each of the several thousand expected pairs of genes, if these latter are single molecules of molecular weight 10^6.[219] In the many-stranded "giant" chromosomes of larval *Drosophila*, there appeared to be among the individual nuclei an

[219] N. B. Kurnick and I. H. Herskowitz, *J. Cellular Comp. Physiol.* **39**, 281 (1952).

enormous range of variation in the degree of replication of the "unit" haploid amount of DNA. One gains the impression that the DNA might, on occasion, be accumulated nonspecifically and stored in the chromosomes in addition to being replicated in specific functional form.

One place in which DNA clearly seems to be accumulated in excess of immediate needs is in the unfertilized egg or early embryo of the sea urchin[220-222] and of the frog.[223,224] Since in the frog embryo the total amount of deoxyribosides does not increase until a late blastula stage,[223] it appears probable that the large amount present in the egg is serving as a storage reserve for early growth, as do other components. The quantity of deoxyriboside present is great enough to more than occupy the whole egg nucleus, and in fact can be demonstrated to be present in the cytoplasm in about 5,000 times the amount present in the haploid sperm nucleus, and yet remains so widely dispersed that it is virtually unrecognizable by staining or photometric assay. Once their own DNA synthesis is well established, embryonic tissues do not show wide departures from the "constant" amount of DNA per cell or per nucleus, except that, as already noted, these rapidly growing tissues may tend to have an average content near the double quantity needed for division. What may be exceptional about the egg DNA is perhaps not so much its reasonable accumulation in high amount, but that it is present in the cytoplasm, and will very likely prove to be in a precursor form, not yet converted into genetically specific DNA.

Preparation of hen's-egg avidin, the biotin-binding protein, usually gives a product containing nondialyzable DNA of typical composition.[225] There is some evidence that the complex of avidin and nucleic acid is not readily dissociable and may exist as such in the egg white.

The kinetoplast of the *Trypanosoma*, a chromatin body lying at the base of the flagellum, takes the Feulgen stain and apparently contains DNA. This body, although not connected with the nucleus, undergoes regular division of its own when the cell divides; it is altogether possible that it may yet prove to have a function in principle not unlike that of the nuclear chromatin. Lwoff includes the kinetoplast among the biological entities endowed with genetic continuity, and states that the Feulgen-positive material appears to be in an annular ring or shell.[226]

[220] C. Vendrely and R. Vendrely, *Compt. rend. soc. biol.* **143,** 1386 (1949).
[221] D. Elson and E. Chargaff, *Experientia* **8,** 143 (1952).
[222] E. Zeuthen, *Pubbl. staz. zool. Napoli* **23,** 47 (1951).
[223] E. Hoff-Jørgensen and E. Zeuthen, *Nature* **169,** 245 (1952).
[224] L. C. Sze, *J. Exptl. Zool.* **122,** 577 (1953).
[225] H. Fraenkel-Conrat and E. D. Ducay, *Biochem. J.* **49,** xxxix (1951).
[226] A. Lwoff, *Colloq. intern. centre natl. recherche sci. (Paris)* **8,** *Unités biol. douées contin. génét.* 7 (1949).

The cytoplasmic factor, kappa, of *Paramecium aurelia* which brings about production of "killer" substance, has been shown in Sonneborn's laboratory to give some of the specific reactions of the DNA-proteins.[227] Since kappa behaves like a self-determining invading particle, although influenced by the host genes, its DNA may be supposed to serve the same presumably genetic type of function as does that of the viruses or *Rickettsiae*. More remarkable, perhaps, is the fact that the "infected" paramecia liberate into their culture fluid a DNA-protein, paramecin, which is the actual substance[228] killing the sensitive animals. The finding that this DNA-protein can bring about the death of those organisms which are not bearing kappa particles, and therefore already producing it, does not at the moment fit simply into the genetic chemical picture of DNA function.

Small DNA-containing bodies have been reported in the cytoplasm of pollen mother cells of several plant species;[229] these bodies are believed to be associated with, or derived from, the nucleus. Somewhat similar bodies are described in the nucleolus-associated chromatin of nerve cells;[207] these may or may not bear a relation to the chromosomes. Leuchtenberger and Schrader[230] have reported that photometrically determined nuclear DNA varied over a range of 30:1 in salivary gland cells of *Helix*, and characteristically and progressively decreased as the secretion product (cytoplasmic polysaccharide) was produced. This would seem to imply a rather direct participation of nuclear DNA in cellular function.

A temporarily elevated DNA content of plant tissues inoculated with virulent crown-gall bacteria is said to parallel the presence of tumor-inducing principle, one of the factors leading to subsequent formation of a crown-gall tumor.[231] These findings are taken to suggest the possible identity of the tumor principle with the new DNA formed in the infected host at that time. As mentioned above, the same workers had reported the transfer of virulence from strain to strain in the bacteria themselves through DNA-containing preparations made from virulent strains.

Preparations of β-glucuronidase from calf spleen, inactivated by dilution, are restored to full activity by DNA preparations.[232] Since depolymerized DNA and, to some extent, PNA are also effective, the possibility that DNA is some sort of coenzyme seems more remote than that it contributes another activator, possibly specific metal ions, to the enzyme system.

[227] J. R. Preer, *Am. Naturalist* **82**, 35 (1948); *Genetics* **33**, 625 (1948).
[228] W. J. van Wagtendonk, *J. Biol. Chem.* **173**, 691 (1948).
[229] A. H. Sparrow and M. R. Hammond, *Am. J. Botany* **34**, 439 (1947).
[230] C. Leuchtenberger and F. Schrader, *Proc. Natl. Acad. Sci. U. S.* **38**, 99 (1952).
[231] R. M. Klein, *Am. J. Botany* **40**, 597 (1953).
[232] P. Bernfeld and W. H. Fishman, *J. Biol. Chem.* **202**, 757 (1953).

IV. Summary—Infection, Heredity, and Infectious Heredity

The deoxypentose nucleic acids are significant participants in those mechanisms whereby the various forms of living matter pass on to their progeny their various specifically organized patterns of growth and metabolism. These substances are prominent constituents of the nuclei of perhaps all cells. Upon some species of bacteria, specific deoxypentose nucleic acids can act even in isolated form as genetic determinants. Their diversity of chemical composition and especially of organization is sufficient to allow for their functioning in countless different specific genetic roles. Furthermore, as cell constituents of remarkable metabolic stability, the deoxypentose nucleic acids are found to increase in amount in close relation to growth. The behavior of this component in cells of the higher forms is organized with nice precision at the successive levels of the nucleus, the chromosome, the linkage group, and the gene. Among the lower orders, the growth of the more exacting parasitic forms involves formation of parasite deoxypentose nucleate more or less directly at the expense of this component of the host cell, and is called an infection. In the case of certain bacterial viruses, such an infection clearly partakes of the nature of an intimate reorganization of the host's genetic mechanisms, induced by deoxypentose-nucleate particles. At this point, the simplest infective processes become analogous to the simplest genetic processes, the bacterial transformations. Both furnish indications that the native deoxypentose nucleate-containing particle is a fundamental biological unit capable of initiating a process which can be viewed under some circumstances as genetic, or under others as infectious.

CHAPTER 28

The Biological Role of the Pentose Nucleic Acids

J. BRACHET

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The biological importance of pentosenucleic acids (PNA) is based on three main lines of evidence: (1) the fact that all plant viruses so far studied are composed of pentosenucleoproteins and that PNA is necessary for their

multiplication; (2) the finding that pentosenucleoproteins, presumably in the form of microsomes, play an important part in embryonic development; and (3) the presence of large amounts of PNA in all cells in which considerable protein synthesis takes place. These three different aspects of the same major problem, i.e., the role of PNA in protein synthesis, will be discussed in the present chapter.

I. The Role of PNA in Plant Viruses

This question has recently been ably reviewed by Markham,[1] who has made important personal contributions in this field.

1. The Composition of Plant Viruses

All the plant viruses which have been purified and, in many instances, crystallized, contain large amounts of PNA, ranging from 6% (tobacco mosaic virus) up to 35% (turnip yellow mosaic virus), in close association with protein. The fact that such relatively simple nucleoproteins are able to reproduce themselves and are thus endowed with genetic continuity is of the utmost importance: plant viruses are ideal material for the study of the synthesis of specific proteins as well as for the solution of fundamental genetic problems. But we shall deal here only with the significance of PNA in plant virus multiplication.

2. The Importance of PNA in Plant Virus Multiplication

a. Abnormal Proteins from Virus-Infected Plants

In their important studies on turnip yellow mosaic virus, Markham and Smith[2] were able by ultracentrifugation to separate two distinct components from crystalline preparations of the virus: both contained serologically identical proteins, but only the more rapidly sedimenting component contained PNA in addition and proved infective When the virus nucleoprotein was introduced into a plant, about 40% of the particles produced were devoid of PNA and incapable of multiplication. These findings led Markham[1] to the very important conclusion that "there is some evidence that the nucleic acid is in fact the substance controlling virus multiplication."

Markham and Smith's[2] findings were soon extended: in 1952, Takahashi and Ishii[3,4] reported the isolation from mosaic-diseased tobacco leaves of an abnormal protein which could be obtained by electrophoresis and which

[1] R. Markham, *2nd Symp. Soc. Gen. Microbiol.* p. 85 (1953); *Advances in Virus Research* **1,** 315 (1953); *Progr. in Biophys. and Biophys. Chem.* **3,** 61 (1953).
[2] R. Markham and K. M. Smith, *Parasitology* **39,** 330 (1949).
[3] W. N. Takahashi and M. Ishii, *Phytopathology* **42,** 690 (1952).
[4] W. N. Takahashi and M. Ishii, *Am. J. Botany* **40,** 85 (1953).

behaved in many ways like tobacco mosaic virus. Simultaneously, Jeener and Lemoine[5,6] discovered a similar (or identical) protein and carried the matter a step further by crystallizing it; this crystallizable protein behaves in the same way as does Markham and Smith's[2] material, i.e., it is immunologically identical with the virus, it is noninfective, and it is free of PNA.

Whether these abnormal proteins present in virus-infected plants are virus precursors, intermediary stages in virus production, or by-products of the virus is not known. It has recently been suggested by Jeener and Lavand'homme[7] that the crystallizable antigen, devoid of PNA, occurring in plants into which tobacco mosaic virus has been injected, represents an intermediary product in virus development which accumulates when the leaves no longer contain enough PNA to make the complete virus. In any case, there is no doubt that proteins serologically very closely related to the plant viruses are noninfective when free of PNA.

b. Interference with Virus Multiplication by Chemical Analogues of Pyrimidines and Purines

If PNA is really essential for the synthesis of plant viruses, one could expect an inhibition of virus multiplication on the addition of substances which interfere with its synthesis: that this is the case has been shown conclusively by Commoner and Mercer,[8,9] who obtained complete inhibition of synthesis of tobacco mosaic virus by thiouracil at a concentration of 4.3×10^{-5} *M*. This inhibition was partially reversed when uracil was added in concentrations of the same order of magnitude.

These findings of Commoner and Mercer[8,9] have been confirmed by Jeener and Rosseels,[10] who recently obtained in addition some quite unexpected results. They found that the inhibition of virus synthesis is greater the smaller the amount of virus present in the leaves to which thiouracil is added. This observation cannot be explained on the basis of a competition between thiouracil in some enzymic reaction during the synthesis of PNA, but rather tends to indicate that thiouracil can be incorporated into the virus PNA and that this incorporation hinders the further multiplication of the modified particles. This interpretation of the facts has been amply confirmed by experiments[10] in which S^{35}-labeled thiouracil was added to leaves infected two days earlier; the concentration of thiouracil was such that the speed of virus multiplication was reduced by 50%. When the virus

[5] R. Jeener and P. Lemoine, *Arch. intern. physiol.* **9,** 547 (1952).
[6] R. Jeener and P. Lemoine, *Nature* **171,** 935 (1953).
[7] R. Jeener and C. Lavand'homme, *Arch. intern. physiol.* **61,** 427 (1953).
[8] B. Commoner and J. Mercer, *Nature* **168,** 113 (1951).
[9] B. Commoner and J. Mercer, *Arch. Biochem. and Biophys.* **35,** 278 (1952).
[10] R. Jeener and J. Rosseels, *Biochim. et Biophys. Acta* **11,** 438 (1953).

was collected and crystallized repeatedly, it was found that it had incorporated the labeled thiouracil in its PNA moiety only, apparently in the form of thiouridylic acid.

Other substances combining with nucleic acids or interfering with their synthesis possess an inhibitory effect on the growth of plant viruses, e.g., trypaflavine (Ryzhkov and Smirnova[11] and basic dyes (Nickell[12]). Recently, R. E. F. Matthews,[13] studying the effects of many analogues of the nucleic acid bases on several plant viruses, found the guanine analogue, 5-amino-7-hydroxy-1-*v*-triazolo-(D)-pyrimidine (guanazolo) to be very effective in the case of lucerne mosaic virus. Since inhibition due to guanazolo is reversed by adenine and guanine, it would appear that the compound acts by blocking the incorporation of guanine into PNA; on the other hand, it might well be that future work with isotopes will show, as in the case of thiouracil, that guanazolo is incorporated as such in the PNA, perhaps leading to the formation of an abnormal PNA and, consequently, to altered virus particles with reduced synthetic abilities.

3. Mechanism of Virus Multiplication

For a further discussion of the mechanism of virus multiplication in relation to protein synthesis, the reader should consult the recent analysis made by Bawden and Pirie,[14] whose main conclusion lies in "the idea that the introduced virus attaches itself to a mechanism concerned with protein synthesis in the host cell and affects it in such a way that thereafter one of the products synthesized is virus." Thus virus multiplication would essentially be a deviation from the normal mechanism of protein synthesis. As Bawden and Pirie[14] point out, it is probably no coincidence that the small plant viruses resemble the sites of protein synthesis in the cell as being rich in PNA, as we shall see later.

II. The Role of PNA in Morphogenesis

This problem has been discussed at some length by the writer, both in a book[15] and in a recent monograph[16] in which more details will be found than can be included in the present review.

[11] V. L. Ryzhkov and V. A. Smirnova, *Microbiology* (*U.S.S.R.*) **17**, 267 (1948); *vide* footnote 13.

[12] G. L. Nickell, *Botan. Gaz.* **112**, 290 (1951).

[13] R. E. F. Matthews, *J. Gen. Microbiol.* **8**, 277 (1953).

[14] F. C. Bawden and N. W. Pirie, *in* "The Nature of Virus Multiplication" (Fildes and Van Heyningen, eds.), p. 21. Cambridge Univ. Press, New York, 1953.

[15] J. Brachet, "Chemical Embryology." Interscience, New York, 1950.

[16] J. Brachet, "Le rôle des acides nucléiques dans la vie de la cellule et de l'embryon." Masson, Paris, 1952.

1. Morphogenetic Gradients and PNA Distribution

Experimental embryology, in order to explain morphogenesis, very often resorts to the gradient concept. The application on a large scale of cytochemical methods for the detection of PNA (mostly the use of basic dyes) has led to the experimental demonstration of the existence of these previously hypothetical gradients in all vertebrate eggs so far studied.

In outline, the distribution of PNA during the early stages of development in the amphibia[15] is as follows: the large oocytes and the unfertilized eggs show a distinct polarity gradient: the amount of PNA decreases steadily and very regularly from the animal to the vegetal pole of the egg.

Fertilization does not modify conspicuously this distribution of PNA, although there are some indications in favor of the view that there is a slight accumulation of this substance in the dorsal half of the egg.

There is little change in this primary polarity gradient during cleavage; but, when morphogenetic movements begin at the time of gastrulation, the dorsal lip of the blastopore (the organizer) is the site of a PNA synthesis. A second PNA gradient, decreasing from dorsal to ventral this time, becomes more and more conspicuous as gastrulation proceeds.

The interaction of these two PNA gradients, the initial animal-vegetal one and the secondary dorso-ventral one, lead at the time of the formation of the nervous system to well-defined cephalo-caudal (in the nervous system and the chorda) and dorso-ventral (chorda and mesoderm) gradients.

Later on, every organ is the site of a PNA accumulation just prior to its differentiation; basophilia, which is an approximate index of the PNA/protein ratio, usually decreases when histological and cytological protein differentiation becomes apparent. It should be added that the existence of the very important animal-vegetal and dorso-ventral PNA gradients at the blastula, gastrula, and neurula stages have been confirmed by direct quantitative analysis: the PNA content of different parts of embryos at these stages has been estimated and found to run parallel with the basophilia gradients (Brachet,[17] Steinert[18]).

In particular, it has been shown by Brachet and Chantrenne[19] that PNA synthesis occurs only in the dorsal half of the embryo during gastrulation and neurulation; this half, in contrast to the ventral region, undergoes extensive morphogenesis, including the induction of the neural tube. In fact, Steinert's[18] extensive estimations of the PNA content during amphibian development conclusively show that there is only little synthesis up to the gastrulation stage; but the increase in PNA becomes considerable as soon as induction starts, i.e., as soon as true morphogenetic processes set in.

[17] J. Brachet, *Enzymologia* **10,** 87 (1941).
[18] M. Steinert, *Bull. soc. chim. biol.* **33,** 549 (1951).
[19] J. Brachet and H. Chantrenne, *Compt. rend. soc. biol.* **140,** 892 (1946).

Recent work with P^{32} by Kutsky[20] also shows that phosphate metabolism is very sluggish during cleavage; but, at gastrulation, there is a transfer of the labile P of ATP to PNA, the specific activity of which markedly increases. This faster uptake of the PNA-phosphorus might well be linked together with inductive processes or increased protein synthesis at that stage.

It should be added, however, that gradients similar to those which have just been described for PNA also occur for other substances: —SH groups linked to the proteins (Brachet[21]), reducing power (Piepho,[22] Fischer and Hartwig,[23] Child,[24]) and alkaline phosphatase (Krugelis[25]). These findings suggest that the morphogenetic gradients are, in fact, gradients in the distribution of cytoplasmic particles (microsomes and mitochondria).

It should be pointed out, finally, that PNA gradients parallel to morphogenetic gradients have also been found in fishes and birds (Brachet,[26] Pasteels,[27] Gallera and Oprecht,[28] etc.), as well as in mammals (Dalcq and Seaton-Jones[29]).

There is thus little doubt that distribution and local synthesis of PNA are important in normal embryonic development: In what way can experiments in which either PNA metabolism or morphogenesis have been artificially altered provide evidence in support of this view?

2. Experimental Modifications of PNA Synthesis or Distribution: Effects on Morphogenesis

It is convenient to deal successively with the effects on PNA synthesis and morphogenesis produced by chemicals (some of which are thought to act specifically on nucleic acids metabolism), by physical agents (heat, centrifugation), and by nuclear abnormalities.

a. Chemical Analogues of Purines and Pyrimidines

It has been shown that chemical analogues of purines and pyrimidines, barbituric acid and benzimidazole, for instance, slow down considerably or even stop completely both morphogenesis and PNA synthesis (Brachet[15]); the effect is perfectly reversible and further development is normal. Similar

[20] P. B. Kutsky, *J. Exptl. Zool.* **115,** 429 (1950).
[21] J. Brachet, *Arch. biol. (Liège)* **51,** 167 (1940).
[22] H. Piepho, *Biol. Zentr.* **58,** 90 (1938).
[23] F. G. Fischer and H. Hartwig, *Z. vergleich. Physiol.* **24,** 1 (1936).
[24] C. M. Child, *J. Exptl. Zool.* **109,** 79 (1948).
[25] E. Krugelis, *Biol. Bull.* **93,** 215 (1947).
[26] J. Brachet, *Arch. biol. (Liège)* **53,** 207 (1942).
[27] J. Pasteels, *Arch. biol. (Liège)* **60,** 235 (1949).
[28] J. Gallera and O. Oprecht, *Rev. suisse zool.* **55,** 243 (1948).
[29] A. Dalcq and A. Seaton-Jones, *Bull. acad. roy. Belg.* **35,** 500 (1949).

results (Brachet[30]) have been obtained with acriflavine, which precipitates nucleic acids *in vitro;* in the latter case, reversibility of development is improved by the addition of PNA or adenylic acid to the blocked embryos.

Bieber *et al.*[31] have recently studied the effects of 25 chemical analogues of purines or pyrimidines on the development of frog's eggs: all inhibited development, but at various stages characteristic for each compound. Inhibitory effects on the development of chick embryos by unnatural synthetic nucleosides have also been reported by Fox and Goodman.[32]

b. Other Toxic Substances

Among the many toxic substances which have been tried on amphibian eggs, only dinitrophenol, usnic acid, and female steroid hormones have been studied from the viewpoint of PNA metabolism.

Dinitrophenol and usnic acid, both of which inhibit the coupling between oxidation and phosphorylation, stop embryonic development completely and reversibly; cytochemically, an increase in the PNA content of the nuclei in these blocked embryos (Brachet[33]) is found; however, chemical estimations show that PNA synthesis is almost entirely blocked while the eggs are in the presence of the poisons and that it is resumed as soon as development is allowed to proceed (Steinert[34]).

Regarding the female sex hormones, Cagianut[35] has reported that stilbestrol and estradiol in appropriate concentrations affect the distribution of PNA in amphibian eggs: the typical gradients are altered and development is highly abnormal. Curiously enough, the addition of yeast PNA works antagonistically and improves development.

c. Physical Agents

Comparable results can be obtained by centrifuging amphibian eggs (Pasteels and Brachet, in Brachet[16]). Centrifugation of eggs just after fertilization leads to marked alterations in the PNA initial polarity gradient and to very abnormal development or even failure of development. The morphological and cytochemical abnormalities induced by such treatment show a close correlation. Centrifugation of blastulæ, on the other hand, leads to the formation of double embryos. Here again, the duplication is linked to the abnormal distribution and local synthesis of PNA.

But the effects of moderate heat shock in developing amphibian embryos

[30] J. Brachet, *Compt. rend. soc. biol.* **140,** 1123 (1946).
[31] S. Bieber, R. F. Nigrelli, and G. H. Hitchings, *Proc. Soc. Exptl. Biol. Med.* **79,** 430 (1952).
[32] J. J. Fox and I. Goodman, *Biochim. et Biophys. Acta* **10,** 77 (1953).
[33] J. Brachet, *Experientia* **7,** 344 (1951).
[34] M. Steinert, *Biochim. et Biophys. Acta* **10,** 427 (1953).
[35] B. Cagianut, *Z. Zellforsch.* **34,** 471 (1949).

are no less impressive in showing the importance of PNA in morphogenesis: if frog gastrulæ are heated at 36.6° for 1 hr., their development is entirely blocked, although they do not cytolyze until 2 to 3 days later (Brachet[36,37]). At a slightly lower temperature (36.2°), a similar heat shock produces only a temporary, reversible block of development. In the first case, cytochemical methods for the detection of PNA indicate that its synthesis is completely arrested, a fact which has been confirmed by the chemical investigations carried out by Steinert.[18] In the case of a reversible shock, PNA synthesis is only temporarily stopped and is resumed as soon as development starts again. It has been further shown (Brachet[36,37]) that, if a fragment of a heated gastrula (irreversibly blocked) is grafted into a normal embryo, it differentiates normally after an initial lag period. As soon as the "revitalization" process sets in, the basophilia of the heated cells begins to increase.

One of the main actions of heat shocks is to affect the microsomes; these undergo a partial denaturation and lose most of their PNA (Brachet[38]). In the heat-treated eggs, a larger proportion of the PNA is no longer bound to the microsomes but is to be found in the supernatant fraction after ultracentrifugation. However, it should be pointed out that the heat shock does not affect the microsomes in a specific manner: the oxygen consumption of the heated embryos is reduced by 30 to 40% (Brachet[38]) and their DNA has already undergone changes in its structure (Thomas[39]), as indicated by an increased ultraviolet absorption.

d. Abnormal Nuclear Composition

A situation essentially similar to that just described prevails in amphibian eggs which have been fertilized by abnormal sperm: hybridization with sperm of a foreign species or fertilization with sperm treated with nitrogen mustard both lead to a highly lethal condition: cleavage of the eggs is perfectly normal, but development ceases entirely at the onset of gastrulation and the blocked lethal hybrids disintegrate a few days later.

We shall deal briefly here with only one of the many lethal hybrids which have been studied, the lethal combination *Rana esculenta* ♀ x *Rana fusca* ♂, because it is the only one in which the behavior of the PNA is fairly well known (Brachet[16,40]).

Just as in the heat-treated eggs, parts of the blocked lethal embryos can be "revitalized" and will develop further if they are grafted in normal

[36] J. Brachet, *Experientia* **4,** 353 (1948).
[37] J. Brachet, *Pubbl. staz. zool. Napoli* **21,** 77 (1949).
[38] J. Brachet, *Bull. soc. chim. biol.* **31,** 724 (1949).
[39] R. Thomas, *Experientia* **7,** 261 (1951).
[40] J. Brachet, *Ann. soc. roy. zool. belg.* **75,** 49 (1944).

gastrulæ: it would appear that the presence of an abnormal nucleus in an egg makes it impossible for the embryo to produce substances which it needs for development, while, in the case of the heated eggs, the high temperature would inactivate morphogenetic substances. But, in both cases, these substances diffuse easily from a normal host into the graft.

In respect of PNA metabolism also, the lethal hybrids behave very much like the heated gastrulæ: there is almost no PNA synthesis in the blocked embryos, while the controls steadily increase their PNA content (Steinert[18]). Also, when a graft of a lethal hybrid is being revitalized under the influence of a normal host, its cells undergo a considerable increase in basophilia.

It may thus be said, in conclusion, that PNA synthesis and embryonic development are so closely linked together that no way has been found so far to dissociate the two processes; there is no doubt either that the integrity of the normally existing PNA gradients is essential for normal morphogenesis.

Let us now consider whether PNA or pentosenucleoproteins, including among them the microsomes, play a special role in the all-important morphogenetic process, neural induction.

3. Pentose nucleoproteins and Neural Induction

It is a well-known fact that the nervous system arises, in normal development, under the *inducing* activity of the underlying organizer (chordomesoblast): presumptive epidermis will differentiate into nervous system if it is placed in contact with the organizer, even when the latter has been killed by heat or alcohol treatment. However, the inductions obtained with killed organizers—or with many tissues coming from a wide variety of animal species—are often less harmonious than the normal ones: for this reason, abnormal inducing agents are often called *evocators* and the reaction which they produce is called an evocation, to distinguish it from a normal induction (for a detailed discussion of these questions, see Needham[41] and Brachet[15]).

The fact that evocators are so widespread in the animal kingdom has led to many attempts to isolate an active "evocating substance." The results have been extremely disappointing, because it was soon apparent that many pure and chemically unrelated substances are equally active. The presumptive epidermis becomes neuralized as an unspecific reaction to many stimuli, very much as the unfertilized egg responds in an unspecific manner to parthenogenetic stimuli. As pointed out by Waddington, Needham, and Brachet,[42] it is very likely that the normal inducing substance

[41] J. Needham, "Biochemistry and Morphogenesis." Cambridge Univ. Press, New York, 1942.

[42] C. H. Waddington, J. Needham, and J. Brachet, *Proc. Roy. Soc.* (*London*) **B120,** 173 (1936).

is already present in the presumptive epidermis, in the form of an inactive complex: evocating agents act in breaking down this inactive complex and in unmasking the active substance. This viewpoint has been further strengthened by Holtfreter's[43] important experiments, showing that neuralization occurs in isolated pieces of presumptive epidermis whenever they undergo a certain type of *sublethal cytolysis:* a shift in the pH of the culture medium, either towards the acid or the alkaline range, is enough to produce spontaneous neuralization in almost all of the ectodermal explants. In more chemical terms, the whole process can be visualized by assuming that the inducing substance is the prosthetic group of some inactive conjugated protein; any agent producing a reversible denaturation of the protein and liberating the prosthetic group will act as an unspecific evocator.

It is obvious, in these circumstances, that the search for a single specific evocating substance becomes a rather hopeless proposition. We shall, however, briefly summarize what is known about PNA as an evocating substance, since this is still a controversial subject.

It has been shown (Brachet[15,44]) that pentosenucleoproteins, especially after alcohol treatment, are very good evocators, whatever their origin or their method of preparation; it was claimed that this evocating activity was markedly decreased if, prior to their implantation into gastrulæ, the nucleoproteins were treated with ribonuclease. But later work in the writer's laboratory (Kuusi,[45-47] Brachet, Gothié, and Kuusi[48]), while confirming that pentosenucleoproteins (tobacco mosaic virus or alcohol-fixed microsomes, for instance) are excellent evocators, failed to substantiate the claim that ribonuclease abolishes the activity of these substances. It is probable that Brachet's[15,44] earlier results were due to proteolytic contaminants in the ribonuclease preparations used: such an explanation is especially likely since it has been shown meanwhile that proteolytic enzymes inactivate various evocators (Toivonen[49]), while blocking the $-NH_2$ groups with formalin (Lallier[50]) or ketene (Kuusi[47]) markedly decreases the evocating activity of various pentosenucleoproteins.

Recently, Engländer *et al.*[51] have confirmed that ribonuclease has little inactivating effect on the evocating power of various tissues; they even

[43] J. Holtfreter, *J. Exptl. Zool.* **106,** 197 (1947).
[44] J. Brachet, *Bull. acad. roy. Belg.* **29,** 707 (1945).
[45] T. Kuusi, *Experientia* **7,** 299 (1951).
[46] T. Kuusi, *Ann. Zool. Soc. Zool. Botan. Fennicae Vanamo* **14,** 1 (1951).
[47] T. Kuusi, *Arch. biol. (Liège)* **64,** 189 (1953).
[48] J. Brachet, S. Gothié, and T. Kuusi, *Arch. biol. (Liège)* **63,** 429 (1952).
[49] S. Toivonen, *Ann. Zool. Soc. Zool. Bot. Fennicae Vanamo* **4,** 28 (1949).
[50] R. Lallier, *Experientia* **6,** 92 (1950).
[51] H. Engländer, A. G. Johnen, and W. Vahs, *Experientia* **9,** 100 (1953).

believe that, after ribonuclease treatment, an increase in the percentage of cephalic inductions can be obtained. It should finally be pointed out that there is no conclusive indication that yeast PNA or mononucleotides can ever induce the neuralization of epidermal explants (Brachet,[16] Kuusi[47]).

These negative and rather discouraging results are of course only what one should expect, now that we know that any agent inducing a condition of sublethal cytolysis will promote the neuralization of ectodermal explants (Holtfreter[43]); they do not mean that pentosenucleoproteins do not play an essential role in normal induction, since sublethal cytolysis certainly induces PNA synthesis. As has been pointed out earlier in this chapter, it is almost certain that they represent, on the contrary, very important morphogenetic factors. The task which chemical embryology will face in the future is to follow the chemical changes undergone by ectoderm subjected either to a normal organizer or to agents producing the sublethal cytolysis condition. In such a study, PNA metabolism, as well as the composition of the microsomes, should remain in the foreground.

The fact that heat shocks, as reported earlier, inhibit inducing processes while they break down the microsomes, suggests that these particles are of special importance in normal induction; in keeping with this suggestion is the fact, discovered recently by Mookerjee,[52] that sublethal cytolysis produced by heat shock does not promote the neuralization of epidermal explants. It should be pointed out, however, that all attempts made to induce the formation of a secondary nervous system by injecting microsomes in the ventral part of a cleaving egg have so far yielded only negative results (Brachet and Shaver,[53] Brachet, Gothié, and Kuusi[48]).

On the other hand, embryologists have often insisted on the similarities existing between normal inductive processes and virus infections (Dalcq,[54] Needham,[41] Brachet,[16] etc.): for instance, once the neural plate has been induced, it becomes in turn an inductor (homeogenetic induction). It is tempting to imagine that the microsomes, which have so much in common with viruses as regards their size and chemical composition, behave like viruses, i.e., as self-duplicating units, capable of spreading from cell to cell (Holtfreter[55]). This hypothesis has already found a certain amount of experimental support (Brachet[56]): for instance, if a cellophane membrane is placed between an organizer and a piece of presumptive epidermis, induction is completely suppressed wherever the ectoderm is protected from the organizer by the membrane. Similar results for the induction of the lens by

[52] S. Mookerjee, *Experientia* **9**, 340 (1953).
[53] J. Brachet and J. R. Shaver, *Experientia* **5**, 204 (1949).
[54] A. Dalcq, "L'oeuf et son dynamisme organisateur." A. Michel, Paris, 1941.
[55] J. Holtfreter, *Symposia Soc. Exptl. Biol.* **2**, 17 (1948).
[56] J. Brachet, *Experientia* **6**, 56 (1950).

the eye-cup have since been reported by McKeehan.[57] They prove conclusively that induction cannot be mediated by simple, easily diffusible, chemical substances; close contact between the organizer and the induced tissue is a prerequisite to successful induction. It has also been found (Brachet[56]) that neutral red, when used as a vital dye for amphibian gastrulæ, stains only the cytoplasmic granules; when, however, a stained explant is intimately joined to an unstained one, the dye diffuses in the latter. Such a diffusion is effectively hindered by the interposition of a cellophane membrane between the explants, although the membrane is permeable to neutral red when in solution.

These findings certainly lend support to the view that normal induction is mediated by large molecular aggregates able to cross cell membranes; they do not prove that the microsomes are involved in the process, but the cytochemical evidence reported earlier in this chapter favors this view.

Summing up, it may be said that there is no doubt that PNA, probably in the form of microsomes, plays an important part in morphogenesis; but the elucidation of the mechanism of its action, especially during neural induction, remains a task for the future.

III. PNA and Protein Synthesis

The conclusion that PNA is somehow concerned with protein synthesis was reached independently by Caspersson[58] and the writer[26] some twelve years ago; it was mainly based on cytochemical results, which have been largely substantiated by direct chemical analysis. After a brief analysis of the cytochemical and biochemical evidence on which the hypothesis rests, we will discuss present ideas about the mechanisms of protein synthesis, first at the cellular, then at the biochemical, level.

1. Cytochemical and Biochemical Evidence for a Link between PNA and Protein Synthesis

a. Cytochemical Evidence

The considerable amount of evidence obtained by Caspersson and his co-workers in favor of the idea that all protein synthesis needs the presence of nucleic acids has recently been summarized in book form by Caspersson;[59] the main results obtained by the writer can be found in his original paper[26] and in two more recent books.[15,16]

Caspersson's results were obtained with his very elegant method of ultraviolet microspectophotometry, while most of the work coming from other laboratories is based on studies made with basic dyes (usually Unna's

[57] M. S. McKeehan, *J. Exptl. Zool.* **117**, 31 (1951).
[58] T. Caspersson, *Naturwissenschaften* **28**, 33 (1941).
[59] T. Caspersson, "Cell Growth and Cell Function." Norton, New York, 1950.

methyl green – pyronine), combined with digestion with ribonuclease. The latter method does not lend itself so well to precise quantitative measurements, but it has the advantage of simplicity and low cost. Both techniques have been discussed in Chapter 17.

It would be a long and almost impossible task to review here all the cytochemical papers in which the intracellular localization of PNA in all possible types of cell has been studied. All we can attempt to do is to give a brief summary of the earlier results which led to the conclusion that PNA plays an essential part in protein synthesis, and to mention a few of the more recent papers supporting the same conclusion.

As was shown first by Caspersson and Schultz,[60] PNA, which had already been demonstrated a few years earlier in the cytoplasm of the sea urchin egg (Brachet[61]), is abundant in rapidly growing cells (onion root-tips, imaginal disks of *Drosophila* larvæ). Proliferating tissues, however, are by no means the only ones to contain large amounts of PNA in their cytoplasm and nucleoli: the same holds true for the exocrine part of the pancreas, the cells producing pepsin in the gastric mucosa, liver cells, nerve cells, young oocytes, and embryos undergoing differentiation, all of which are the site of marked protein synthesis. On the other hand, many tissues which have a very high physiological activity, but which do not synthesize large amounts of protein, contain only small amounts of PNA: such is the case for heart, muscle, or kidney (Caspersson,[58] Caspersson, Landström-Hydén and Aquilonius,[62] Caspersson and Thorell,[63] Hydén,[64,65] Brachet[26]). Microorganisms, which multiply very rapidly and thus synthesize very quickly their own proteins (e.g., yeasts or bacteria), are also very rich in PNA (Caspersson and Brandt,[66] Malmgren and Hedén[67]).

It is already obvious from this very brief description that all the organs which synthesize large amounts of proteins, whether for growth or multiplication, are always rich in PNA, which is localized in the nucleolus and the cytoplasm; all other cells and tissues have a much lower content in PNA and much less conspicuous nucleoli.

Further confirmatory evidence may also be cited: one of the organs which has the largest PNA content is the silk gland in silkworms (Brachet,[17] Denucé[68]), the only known function of which is the production of the pro-

[60] T. Caspersson and J. Schultz, *Nature* **142,** 294 (1938).
[61] J. Brachet, *Arch. biol. (Liège)* **44,** 519 (1933).
[62] T. Caspersson, H. Landström-Hydén, and L. Aquilonius, *Chromosoma* **2,** 111 (1941).
[63] T. Caspersson and B. Thorell, *Chromosoma* **2,** 132 (1941).
[64] H. Hydén, *Z. mikroskop. anat. Forsch.* **54,** 96 (1943).
[65] H. Hydén, *Acta Physiol. Scand.* **6,** Suppl. 17 (1943).
[66] T. Caspersson and K. Brandt, *Protoplasma* **35,** 507 (1941).
[67] B. Malmgren and C. G. Hedén, *Acta Pathol. Microbiol. Scand.* **24,** 437, 448, 472, 496 (1948).
[68] J. M. Denuce, *Biochim. et Biophys. Acta* **8,** 111 (1952).

tein silk. During blood cell formation, there is a steady decrease of the PNA content partly associated with the endocellular synthesis of hemoglobin (Thorell[69]). While endocrine glands are relatively poor in PNA, it is a striking fact that stimulation of hormonal secretion in the pituitary is linked with a marked increase in the PNA content (Desclin,[70] Herlant[71]); more recently, Abolins[72] also concluded that hormone synthesis in the anterior pituitary is related to the amount of PNA present in the cells. In the liver, the PNA content markedly decreases in fasting animals, a fact which will be discussed later.

There is thus no doubt that the PNA content of various cells can be made to vary under different physiological conditions, but always in relation to protein synthesis. Some still more recent work points towards the same direction: during spermatogenesis, protein and PNA synthesis are closely linked together, while DNA synthesis is independent of protein increase (Schrader and Leuchtenberger[73]). In the salivary gland of *Drosophila* larvæ, PNA content is directly related to the intensity of secretion: both increase or decrease together (Lesher[74]). According to Rabinovitch *et al.*,[75,76] a positive correlation between PNA content and protein synthesis is found in the seminal vesicles of rats injected with testosterone propionate, as well as in the salivary glands after ligature of the excretory ducts. Finally, in the giant unicellular alga *Acetabularia mediterranea*, the PNA content of the nucleolus markedly decreases when the organism stops growing after it has been left in the dark for some weeks (Stich[77]).

This series of examples, which is by no means exhaustive, should suffice to prove that we are really dealing with a very general phenomenon, occurring in all living organisms. We shall now find out whether the cytochemical evidence is confirmed by quantitative chemical analyses of PNA from different biological sources.

b. Quantitative Evidence

A large number of independent investigations, which could hardly be completely and adequately reviewed here, show clearly that there is a good

[69] B. Thorell, "Studies on the Formation of Cellular Substances during Blood Cell Production." H. Kimpton, London, 1947.
[70] L. Desclin, *Compt. rend. soc. biol.* **133,** 457 (1940).
[71] M. Herlant, *Arch. biol. (Liège)* **54,** 225 (1943).
[72] L. Abolins, *Exptl. Cell Research* **3,** 1 (1952).
[73] F. Schrader and C. Leuchtenberger, *Exptl. Cell Research* **1,** 421 (1950).
[74] S. Lesher, *Exptl. Cell Research* **2,** 577 (1951).
[75] M. Rabinovitch, L. C. U. Junqueira, and H. A. Rothschild, *Science* **114,** 551 (1951).
[76] M. Rabinovitch, H. A. Rothschild, and L. C. U. Junqueira, *J. Biol. Chem.* **194,** 835 (1952).
[77] H. Stich, *Z. Naturforsch.* **6b,** 319 (1951).

correlation between the basophilia or ultraviolet absorption of different tissues and their PNA content. This parallelism was already made apparent by Brachet's[17] earlier estimations of the PNA content of various tissues; subsequently, different and better methods have been devised for quantitative estimations of PNA, without altering the initial conclusions. Extensive reviews of the whole question have been given by Davidson,[78,79] who has made important experimental contributions to the subject: Davidson[79] comes to the conclusion that the nucleic acid content of different tissues, as determined by chemical methods, is generally in accordance with the values which might be expected on histological grounds.

Thus, it will be no surprise to learn that glandular organs, synthesizing large amounts of proteins, such as pancreas, salivary glands, and gastric and intestinal mucosæ, are rich in PNA; that the same is true, to a somewhat lesser extent, for organs where mitoses are frequent (spleen, thymus, lymph nodes, testis, various tumors); and that kidney, brain, heart, and lung have a much lower PNA content. This question has already been discussed in Chapter 16.

Biochemical work on embryonic material from Davidson's laboratory[80,81] has quantitatively demonstrated yet another cytochemical finding (Thorell[69]): synthesis of PNA always precedes protein synthesis, so that a rising protein content is characteristic of embryonic differentiation. This conclusion is perfectly in keeping with the cytochemical work done on amphibian embryos, which has been discussed earlier in this review. It has also been found by Mandel *et al.*[82] that during development of the brain in chick embryos, synthesis of PNA is linked to protein synthesis, while DNA synthesis is related to nuclear multiplication.

Another interesting case is to be found in liver, where fasting or administration of a protein-poor diet is followed by a decrease in basophilia and a parallel drop in the actual PNA content (Davidson[78]). In contrast to this, the DNA content of the liver is not affected when the protein content of the diet is altered (Mandel *et al.*,[83] Campbell and Kosterlitz[84]). Campbell and Kosterlitz[85] finally draw the conclusion that "the PNA content of a unit of liver cells is determined mainly by the protein content of the diet," an opinion which is also shared by Munro *et al.*;[86] these workers recently dis-

[78] J. N. Davidson, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 50 (1947).
[79] J. N. Davidson, "The Biochemistry of Nucleic Acids." Methuen, London, 1950.
[80] J. N. Davidson, I. Leslie, and C. Waymouth, *Biochem. J.* **44,** 5 (1949).
[81] I. Leslie and J. N. Davidson, *Biochim. et Biophys. Acta* **7,** 413 (1951).
[82] P. Mandel, R. Bieth, and R. Stoll, *Bull. soc. chim. biol.* **31,** 1335 (1949).
[83] P. Mandel, M. Jacob, and L. Mandel, *Bull. soc. chim. biol.* **32,** 80 (1950).
[84] R. M. Campbell and H. W. Kosterlitz, *Endocrinology* **6,** 308 (1950).
[85] R. M. Campbell and H. W. Kosterlitz, *Biochim. et Biophys. Acta* **8,** 664 (1952).
[86] H. N. Munro, D. J. Naismith, and T. W. Wakramanayake, *Biochem. J.* **54,** 198 (1953).

covered that when the basal diet contains protein, addition of energy in the form of either carbohydrate or fat results in a marked increase in the PNA content of the whole liver, while, when the diet lacks protein, an increase in energy intake causes only very small changes in the amount of PNA.

The existence of a close quantitative relationship between PNA content and protein synthesis is particularly impressive in growing cultures of microorganisms: bacteria, which undergo a very rapid synthesis of their own proteins during growth, are extremely rich in PNA: values up to 11.5% dry weight have been reported by Vendrely.[87]

Recent work carried out in several different laboratories shows an excellent correlation between the synthesis of PNA and the synthesis of proteins, if bacterial growth is studied during the logarithmic phase: for instance, Caldwell *et al.*[88] find the PNA content of bacteria to be proportional to the growth rate, whatever the experimental conditions (changes in the nitrogen source of the culture medium, presence or absence of inhibitors, normal organisms, or slow growing mutants). Similar findings have been reported recently by Northrop,[89] Wade,[90] and Price:[91] the latter not only finds the synthesis of PNA and proteins to be parallel during the logarithmic phase of growth, but he also reports that when adaptative synthesis for the utilization of lactose occurs, PNA increases simultaneously with the protein.

A still more recent study by Gale and Folkes[92] shows that *Staphylococci* synthesize protein in the presence of glucose and amino acids; if purines and pyrimidines are added to this medium, nucleic acids are also synthesized. But it is a very interesting fact that, if the medium contains no amino acids, there is no nucleic acid synthesis; furthermore, the presence of purines and pyrimidines in the medium enhances protein synthesis. There thus exists a strong positive correlation between the nucleic acid content of the cells and the rate of protein synthesis; if the nucleic acid content falls below 4%, protein synthesis stops completely.

Gale and Folkes[93] have further found that protein synthesis and PNA synthesis can, however, be dissociated by the use of antibiotics: for instance, chloromycetin, aureomycin, and terramycin inhibit protein synthesis, but increase nucleic acid synthesis. Similar results, also in bacteria, had already

[87] R. Vendrely, "Un symposium sur les protéines." Masson, Paris, 1946.
[88] P. C. Caldwell, E. L. Mackor, and Sir Cyril Hinshelwood. *J. Chem. Soc.* **1950,** 3151.
[89] J. H. Northrop, *J. Gen. Physiol.* **36,** 581 (1953).
[90] H. E. Wade, *J. Gen. Microbiol.* **7,** 24 (1952).
[91] W. H. Price, *J. Gen. Physiol.* **35,** 741 (1952).
[92] E. F. Gale and J. P. Folkes, *Biochem. J.* **53,** 483 (1953).
[93] E. F. Gale and J. P. Folkes, *Biochem. J.* **53,** 493 (1953).

been reported by Levy *et al.*:[94] addition to growing *Proteus* of 2×10^{-3} *M* cobaltous sulfate stops growth without arresting PNA synthesis. Miura and his co-workers[95,96] have also reported comparable findings when bacteria are treated with the antibiotic usnic acid. The significance of these observations, which have all been made in the presence of antibiotics which interfere with normal growth of the microorganisms, will be discussed in a later section.

The very great importance of the culture conditions in such experiments cannot be overemphasized; as was shown very clearly by Jeener,[97,98] the relationships between PNA content and protein synthesis are very different in the case of the flagellate *Polytomella cæca*, whether one is dealing with a continuous culture (in the exponential phase of growth) or not: if one compares cells during the various stages of growth of a culture, there is no linear relationship between the quantity of PNA per milligram protein nitrogen and the rate of protein synthesis (Jeener[97]). If, on the other hand, continuous cultures are used under conditions where growth maintains itself during long periods at a constant rate which can be varied at will between wide limits, a strict relationship is found between the rate of protein synthesis and the quantity of PNA in excess of a constant basal figure always present in the cells. Thus the close relationship between the quantity of PNA present and protein synthesis exists only for systems in the steady state; when the physiological conditions of the cells are changing rapidly, for instance at the end of the lag and logarithmic phases of growth, no such simple correlation can be found (Jeener[98]).

These conclusions are by no means unexpected if one recalls the aforementioned cytochemical findings of Malmgren and Hedén:[67] using Caspersson's ultraviolet absorption technique, they found the absorption to be very low in an 18-hr. agar culture of *B. cereus*. There is a moderate increase in the absorption due to PNA during the lag phase. However, during the logarithmic phase of growth the ultraviolet absorption is very intense and the PNA content then reaches its peak. It finally falls to the original low level and all divisions cease at the same time—cell division is thus, in this case, very clearly and closely related to the PNA content.

c. Additional Evidence

Beside cytochemical and quantitative evidence indicating a role of PNA in protein synthesis additional evidence, although of a more circumstantial nature, should be briefly mentioned.

[94] H. B. Levy, E. T. Skutch, and A. L. Schade, *Arch. Biochem.* **24,** 198 (1949).
[95] Y. Miura and Y. Nakamura, *Bull. soc. chim. biol.* **33,** 1409 (1951).
[96] Y. Miura, Y. Nakamura, and H. Matsudaira, *Bull. soc. chim. biol.* **33,** 1577 (1951).
[97] R. Jeener, *Biochim. et Biophys. Acta* **8,** 125 (1952).
[98] R. Jeener, *Arch. Biochem. and Biophys.* **43,** 381 (1953).

For instance, H. and R. Jeener[99] have been able, in the case of *Thermobacterium acidophilus*, to interfere selectively with either PNA or DNA synthesis by the removal from the culture medium of uracil and DNA, respectively. In the absence of DNA, the cells still grow as elongated filamentous forms, but the number of bacterial nuclei remains small. In cultures deprived of uracil, growth is inhibited and both nuclei and cytoplasm are affected. These findings indicate that while protein synthesis is dependent on PNA synthesis, it is much less directly related to DNA synthesis.

Another indirect line of evidence can be found in Swenson's[100] interesting discovery that, when ultraviolet light inhibits the adaptive synthesis of galactozymase in yeasts, the action spectrum is very similar to a nucleic acid absorption spectrum; it bears no relation to the absorption spectrum of an unconjugated protein. As pointed out by Swenson,[100] "the results strongly indicate that nucleic acid is the cellular constituent affected by the light." Swenson's results, however, do not allow us to decide whether the light-sensitive substance is PNA rather than DNA. Indeed, recent work by Kelner[101] shows that while ultraviolet light at 253.7 mμ has little immediate effect on growth and PNA synthesis, both phenomena being affected in the same way, it seems to inhibit DNA synthesis immediately and in a specific manner. It is therefore possible that the adaptive synthesis of galactozymase is under direct nuclear control, although such a conclusion is hard to reconcile with a number of facts which will be discussed later.

A recent observation by Jeener[102] may also prove of great importance. It was mentioned earlier that Jeener and Rosseels[10] found labeled thiouracil to be incorporated into the PNA of tobacco mosaic virus, when the leaves are treated with this radioactive chemical analogue of uracil. When tobacco leaves showing a systemic infection by tobacco mosaic virus are treated with ordinary thiouracil, it is found that there is a considerable and parallel decrease in the turnover rates of the glutamic acid present in the proteins of the leaves and of the virus, and in the purines and pyrimidines from normal and virus PNA's. The fact that the turnovers of both the PNA and the proteins are affected simultaneously constitutes still more evidence in favor of an intimate relationship between PNA and protein synthesis.

It should finally be mentioned that, according to Okamoto[103] and Bernheimer,[104] addition of PNA from several but not all sources greatly stimu-

[99] H. Jeener and R. Jeener, *Exptl. Cell Research* **3,** 675 (1952).
[100] P. A. Swenson, *Proc. Natl. Acad. Sci. U. S.* **36,** 699 (1950).
[101] A. Kelner, *J. Bacteriol.* **65,** 252 (1953).
[102] R. Jeener, personal communication.
[103] H. Okamoto, *Japan. J. Med. Sci. IV, Pharmacol.* **12,** 167 (1939).
[104] A. W. Bernheimer, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 358. Johns Hopkins Press, Baltimore, 1952.

lates the production of the hemolytic toxin called *streptolysin S* during streptococcal growth: the precise chemical nature of streptolysin S is not yet known, but enzymic digestion experiments show that protein is essential for activity (Bernheimer[105]).

2. Cellular Mechanisms of Protein Synthesis

a. Short Summary of Caspersson's Theory

In 1940, T. Caspersson[58,59,106] proposed a general theory of the mechanism of protein synthesis in the intact cell. This theory, as will be seen later, still holds good, although some of its aspects should now undergo modification.

Caspersson's theory is based on three fundamental principles:[106]

1. All protein synthesis requires the presence of nucleic acids.
2. Quantitatively the most important nucleic acids in the chromosomes are of the deoxyribose type.
3. The nucleus itself is a cell organelle especially organized as the main center for the formation of proteins.

Starting from these premises, Caspersson[58,59] suggests that the euchromatin, genetically active and rich in DNA, controls the synthesis of the more complex and specific proteins: these would thus be products of the genes. Heterochromatin, especially the nucelolus-associated chromatin, controls the synthesis of histone-like proteins, which are rich in diamino acids. These substances accumulate to form the main bulk of the nucleolus. From the nucleolus, the basic proteins diffuse towards the nuclear membrane, cross it, and induce in the perinuclear cytoplasm an intensive production of pentosenucleoproteins, the basic amino acids (arginine and histidine) being precursors of the PNA purines. These cytoplasmic pentosenucleotides somehow induce the synthesis of cytoplasmic proteins. As indicated in Fig. 1, nucleoli and cytoplasmic PNA are intermediaries between nucleolar-associated chromatin and cytoplasmic proteins.

Certain aspects of Caspersson's initial theory, which is now more than twelve years old, can hardly be retained at present: this is especially true for the role which the histones are supposed to play. Work by Mirsky and Pollister[107] and by Mirsky[108] has shown that the absorption spectra of purified histones cannot be distinguished from those of more complex proteins, e.g., albumins. Furthermore, Vincent,[109] working with nucleoli isolated from starfish oocytes, did not succeed in obtaining any histone-like

[105] A. W. Bernheimer, *J. Exptl. Med.* **90,** 373 (1949).
[106] T. Caspersson, *Symposia Soc. Exptl. Biol.* **1,** 127 (1947).
[107] A. E. Mirsky and A. W. Pollister, *Proc. Natl. Acad. Sci. U. S.* **28,** 344 (1942).
[108] A. E. Mirsky, *Advances in Enzymol.* **3,** 1 (1943).
[109] W. S. Vincent, *Proc. Natl. Acad. Sci. U. S.* **38,** 139 (1952).

protein from this material. Finally, recent work, reviewed in Chapter 23 dealing with the biosynthesis of purines, has shown that the purine ring is preferentially sunthesized from simple precursors such as carbon dioxide, formate, and glycine; it does not arise directly from arginine and histidine, as was believed when Caspersson worked out his theory in 1940.

It should also be recalled that in those days very little was known about the existence of various cytoplasmic fractions, all of them containing PNA: it is therefore not surprising that no reference is to be found, in Caspersson's theory, to microsomes, mitochondria, or cell sap PNA.

The relationship existing between these various types of particles is the first problem to be discussed here: we shall then consider the origin of

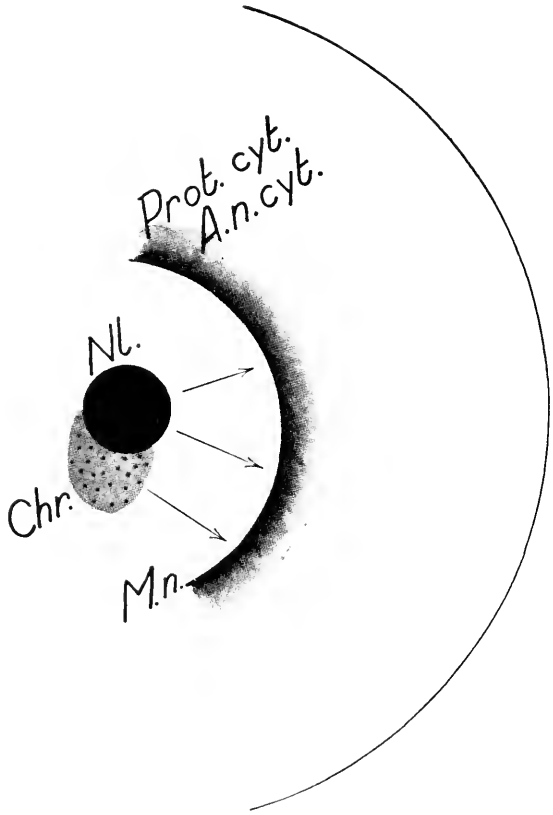

FIG. 1. Diagrammatic view of the cytoplasmic protein-forming system. Nl: nucleolus; Chr: nucleolus-associated chromatin; M.n.: nuclear membrane; A.n.cyt.: cytoplasmic PNA; Prot. cyt.: cytoplasmic proteins. The arrows indicate the migration of proteins from the heterochromatin, direct or via the nucleolus, to the nuclear membrane (after Caspersson[106]).

cytoplasmic PNA and its relationship to nuclear PNA, a problem directly bearing on Caspersson's theory. We shall subsequently discuss another aspect of the same theory, viz., the role played by the nucleus in protein synthesis. Finally, the significance of the microsomes for protein synthesis will be examined in more detail.

b. New Facts since Caspersson's[58] *Theory*

(1) *Presence of PNA in Different Cytoplasmic Particles.* The chemical composition of the various cytoplasmic particles has already been dealt with in Chapter 21. It is enough for our present purpose to recall that PNA is present in all cellular fractions which can be obtained by differential centrifugation (nuclei, mitochondria, microsomes, and cell sap).

In the nuclei, PNA is probably associated mainly with the nucleolus (see Chapter 18). While in most cases the PNA content of isolated nuclei is rather low (*ca.* 1.5% according to Allfrey *et al.*[110]), it can reach the same value as in the cytoplasm in rapidly growing tissues, e.g., wheat germ (Stern and Mirsky[111]). While mitochondria are rather poor in PNA, the amount present in the microsomes can exceed 30% in embryonic material: there is little doubt that most of the basophilic or ultraviolet-absorbing substances which have been studied by the cytochemists are in fact microsomes or aggregates of microsomes. Nonsedimentable (cell sap) PNA is especially abundant in rapidly growing cells (Brachet and Jeener[112]) such as yeast cells and amphibian or chick embryos. It is probable that this soluble fraction has something to do with cell division, as indicated by its definite increase in hepatomas (Price *et al.*[113]).

The possible relationships between microsomes and mitochondria (see Chapter 21) are still very much open to discussion: in the opinion of Claude,[114,115] the original discoverer of the microsomes, both fractions are entirely independent of each other; evidence in favor of this view can be found in Claude's[115] demonstration that, in ultracentrifuged liver cells, the mitochondrial and basophilic (presumably microsomal) layers are well separated. On the other hand, D. Green[116] believes that microsomes are little more than breakdown products of mitochondria, an opinion which is hard to reconcile with the fact that microsomes and mitochondria are very

[110] V. Allfrey, H. Stern., A. E. Mirsky, and H. Saetren, *J. Gen. Physiol.* **35,** 529 (1952).

[111] H. Stern and A. E. Mirsky, *J. Gen. Physiol.* **36,** 181 (1952).

[112] J. Brachet and R. Jeener, *Enzymologia* **11,** 196 (1944).

[113] J. M. Price, J. A. Miller, E. C. Miller, and G. M. Weber, *Cancer Research* **9,** 103 (1949).

[114] A. Claude, *Science* **87,** 467 (1938).

[115] A. Claude, *Biol. Symposia* **10,** 111 (1943).

[116] D. E. Green, *2nd Intern. Congr. Biochem., Paris* p. 1 (1952).

different in their PNA content and enzymic constitution (Chantrenne,[117] Barnum and Huseby,[118] Huseby and Barnum[119]). According to Chantrenne,[117] all intermediate stages can be found between very small microsomes and large mitochondria, and it would appear that the latter grow out of microsomes by a process of progressive complication.

More recently, however, Novikoff *et al.*[120] have separated 8 different fractions from liver homogenates and concluded that 3 of them were purely mitochondrial and 2 entirely microsomal; the remaining 3 were mixtures of mitochondria and microsomes. They conclude that the cell particles isolated by differential centrifugation are mitochondria, and large and small microsomes. A somewhat similar opinion is expressed by Smellie *et al.*,[121] who find by electron microscopy of tissue extracts only mitochondria and microsomes, the latter being less homogeneous in appearance than the former.

One recent finding which is in agreement with Chantrenne's[117] suggestion that microsomes might represent early stages in the development of mitochondria is the fact that, in rat liver at least, the PNA's in both types of particle and in the nonsedimentable fraction (cell sap) have the same composition in terms of molar proportions of bases (Elson and Chargaff,[122] Crosbie *et al.*[123]). In this respect, they differ markedly from nuclear PNA, a fact which will be discussed in the next section.

However, results on the incorporation of various labeled precursors in the PNA of the different cellular fractions do not, on the whole, agree with Chantrenne's[117] hypothesis. According to this hypothesis, microsomes should show a higher specific activity than mitochondria, in short-duration experiments at any rate. Such results have been reported by Jeener and Szafarz[124] in rat liver with P^{32}; within the cytoplasm, the specific radioactivity decreases progressively from the supernatant to the mitochondria, the microsomes giving intermediate values. In growing tissues (mouse embryos), however, the mitochondria have an activity almost as high as that of the supernatant.

Jeener and Szafarz's[124] results with liver are similar to those reported a little earlier by Marshak and Calvet,[125] who also found a higher specific

[117] H. Chantrenne, *Biochim. et Biophys. Acta* **1**, 437 (1947).
[118] C. P. Barnum and R. A. Huseby, *Arch. Biochem.* **19**, 17 (1948).
[119] R. A. Huseby and C. P. Barnum, *Arch. Biochem.* **26**, 187 (1950).
[120] A. B. Novikoff, E. Podber, J. Ryan, and E. Noe, *Federation Proc.* **11**, 265 (1952).
[121] R. M. S. Smellie, W. M. McIndoe, R. Logan, and J. N. Davidson, *Biochem. J.* **54**, 280 (1953).
[122] D. Elson and E. Chargaff, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 329. Johns Hopkins Press, Baltimore, 1952.
[123] G. W. Crosbie, R. M. S. Smellie, and J. N. Davidson, *Biochem. J.* **54**, 287 (1953).
[124] R. Jeener and D. Szafarz, *Arch. Biochem.* **26**, 54 (1950).
[125] A. Marshak and F. Calvet, *J. Cellular Comp. Physiol.* **34**, 451 (1949).

activity for microsomes than for mitochondria. On the other hand, Barnum and Huseby[126] found practically no difference between the two fractions in this respect, and this observation has been confirmed by recent work from Davidson's laboratory (Smellie *et al.*[121]).

The same group (Smellie *et al.*[127]) has studied the incorporation of two other labeled precursors, glycine-N^{15} and formate-C^{14}, into the PNA of various cytoplasmic fractions: in confirmation of their earlier results with P^{32}, they found no great difference between mitochondria and microsomes. The supernatant (cell sap) fraction showed a higher activity than the particulate fractions (mitochondria and microsomes); but, as Smellie *et al.*[121] have been careful to point out, the possibility cannot be excluded that this fraction is contaminated with PNA of nuclear origin, with a high specific activity, which might leach out of nuclei during the experimental procedure.

It should finally be mentioned that work with P^{32} by Jeener[128] on the flagellate *Polytomella cœca*, has led him to conclusions which are not compatible with Chantrenne's[117] hypothesis: the experiments indicate that there are probably, as Chantrenne believed, more than two types of particle, the mitochondria and the microsomes. But the view that the smallest particles serve as nuclei for the synthesis of the larger is not confirmed by the experimental results, which show that the quantity of PNA synthesized at any instant of time is proportional to the quantity of PNA present. No significant difference in specific activity is to be found between microsomes and mitochondria in these experiments.

The fact that, in *Polytomella*, the quantity of PNA synthesized at any moment is proportional to the quantity of PNA present leads Jeener[128] to the interesting conclusion that this relationship can be best explained by assuming that all types of ribonucleoprotein particles, whatever their size, are capable of independent autocatalytic multiplication.

Such a hypothesis is certainly in agreement with most, if not all, the experimental work done with radio-isotopes on cell particles. It would now appear that microsomes and mitochondria are independent units; their syntheses inside the cell go on side by side and might well be the result of some autocatalytic multiplication process. The question which now arises is whether this synthesis of the cytoplasmic particles is limited by the production of PNA in the nucleus and its diffusion in the cytoplasm.

(2) *Interrelations between Nuclear PNA and Cytoplasmic PNA.* Caspersson's theory was largely based on the observations made in his laboratory that an accumulation of ultraviolet-absorbing material, rich in PNA, took

[126] C. P. Barnum and R. A. Huseby, *Arch. Biochem.* **29**, 7 (1950).
[127] R. M. S. Smellie, W. M. McIndoe, and J. N. Davidson, *Biochim. and Biophys. Acta* **11**, 559 (1953).
[128] R. Jeener, *Biochim. et Biophys. Acta* **8**, 270 (1952).

place around the nuclear membrane (Caspersson,[59,106] Hyden[64,65]); similar observations, made with basic dyes, have been reported in several laboratories on various types of cell (see, for instance, Barr and Bertram,[129] Lagerstedt,[130] Brachet[131]). There is little doubt that the presence of large amounts of PNA, presumably in the form of microsomes surrounding the nuclear membrane, is a general phenomenon in all protein-synthesizing cells.

Biochemical investigations with labeled isotopes as precursors of PNA lend some support to Caspersson's interpretation of these cytochemical findings: Marshak[132] was the first to report that the specific radioactivity of the nuclear PNA, studied with P^{32}, is much greater than that of the cytoplasmic PNA. He considered that the nuclei contain a special type of nucleic acid which is neither PNA nor DNA, but has some properties common to each and which is a precursor to cytoplasmic PNA. Somewhat later, Marshak and Calvet[125] expressed the opinion, on the basis of experiments in which the specific activity of P^{32} in PNA was measured in isolated nuclei and microsomes, that nuclear PNA behaves as a precursor to cytoplasmic PNA (see also Chapter 26).

The higher specific activity of nuclear PNA labeled with P^{32} as compared with cytoplasmic PNA has been confirmed by Jeener and Szafarz,[124] by Barnum and Huseby,[126] and by Smellie *et al.*[121] All agree that their experimental findings are compatible with the possibility that nuclear PNA is the precursor of cytoplasmic PNA: but, while Jeener and Szafarz[124] take this explanation as the most probable one, Barnum and Huseby[126] emphasize that this conclusion is made much less likely by the fact that, in their experiments, the relative specific activity of the supernatant PNA remains a constant percentage of the relative specific activity of the nuclear PNA. It should be noted, however, that Barnum and Huseby's[126] findings could be easily explained if some of the PNA present in the supernatant fraction were derived from the nuclei, a possibility which Smellie *et al.*[121] do not rule out. In any case, Barnum and Huseby[126] suggest an alternative explanation: some unidentified intermediate, which could be of either nuclear or cytoplasmic origin, is the immediate precursor of both nuclear PNA and part of the cytoplasmic PNA.

Another interesting point is made by Smellie *et al.*:[121] while their results do not preclude the possibility that nuclear PNA is the ultimate precursor of cytoplasmic PNA, the activity–time curves are not consistent with a process of simple diffusion from the nucleus into the cytoplasm. They further point out that, if simple diffusion occurred, cytoplasmic PNA and

[129] M. Barr and E. Bertram, *Nature* **163,** 676 (1949).
[130] S. Lagerstedt, *Acta Anat. Suppl.*, **9,** 1 (1949).
[131] J. Brachet, *Compt. rend. soc. biol.* **143,** 1300 (1949).
[132] A. Marshak, *J. Cellular Comp. Physiol.* **32,** 381 (1948).

nuclear PNA should have the same molar composition: we have already seen that this is not the case (Elson and Chargaff,[122] Crosbie *et al.*,[123] Marshak[133]).

Work on the same lines and leading to similar conclusions has been done with precursors labeled with other isotopes; the differences between nuclear PNA and cytoplasmic PNA are, however, usually smaller than those found with P^{32}. Using labeled glycine, Bergstrand *et al.*[134] found much larger quantities of N^{15} incorporated into the purines of nuclear PNA than into the cytoplasmic fractions. Similar results have been obtained by Payne *et al.*,[135] who used formate-C^{14}, by Anderson and Aquist[136] for orotic acid-N^{15}, and, finally, by Smellie *et al.*,[127] who compared P^{32}, glycine-N^{15}, and formate-C^{14}: in all these experiments, nuclear PNA showed a high rate of incorporation which exceeded that of the PNA of all cytoplasmic fractions.

Mention should also be made of a paper by Hurlbert and Potter,[137] who studied in the rat the fate of labeled orotic acid which behaved as a highly specific precursor for PNA pyrimidines. They found that, at the beginning of the experiment, the specific activity of nuclear PNA is higher than that of cytoplasmic PNA, but that it levels off later on. Once again, the experimental results are compatible with the view that nuclear PNA is a precursor of cytoplasmic PNA; but the possibility that both are being synthesized independently, at different rates, cannot be excluded. These problems are also discussed in Chapter 26.

Nothing is as yet known about the metabolic pathways of PNA synthesis in the nucleus and in the cytoplasmic particles; but, it might be significant that work done in Mirsky's laboratory (Stern *et al.*[138]) has shown that the only enzymes which are found in high proportions in most nuclei are adenosine deaminase, nucleoside phosphorylase, and guanase, i.e., enzymes related to nucleoside or purine metabolism. Many other hydrolyzing enzymes, as well as catalase, were also studied, but none of them was found in such high concentrations in isolated nuclei. Such an enzyme distribution suggests an important role of the cell nucleus in the synthesis of nucleotides and possibly of the nucleic acids themselves: this conclu-

[133] A. Marshak, *J. Biol. Chem.* **189,** 607 (1951).

[134] A. Bergstrand, N. A. Eliasson, B. Norberg, P. Reichard, and H. von Ubisch, *Cold Spring Harbor Symposia Quant. Biol.* **13,** 22 (1948).

[135] A. H. Payne, L. S. Kelly, G. Beach, and H. B. Jones, *Cancer Research* **12,** 5426 (1952).

[136] E. P. Anderson and S. E. G. Aquist. *2nd Intern. Congr. Biochem., Paris* p. 197 (1952).

[137] R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.* **195,** 257 (1952).

[138] H. Stern, V. G. Allfrey, A. E. Mirsky, and H. Saetren, *J. Gen. Physiol.* **35,** 559 (1952).

sion is also borne out by the recent experiments of Hogeboom and Schneider,[139] showing that the enzyme catalyzing the synthesis of DPN from nicotinamide mononucleotide and ATP is largely, if not exclusively, confined to the cell nucleus in the liver.

Finally, another type of experiment of a more biological nature leads to the same conclusion. When amœbæ (*Amœba proteus*) are cut into two parts, only one part retains the single nucleus. In the absence of any external food supply, the nucleated half survives for about 3 weeks, while the nonnucleated part dies after 2 weeks. Cytochemical staining methods have shown that the basophilia of the nonnucleated half drops markedly 3 to 4 days after the operation and that it becomes very weak after 10 days (Brachet[140,141]): as early as the 4th or 5th day after enucleation, the difference between the two types of fragments is very striking. Microchemical analysis of the PNA content of both halves has yielded results which are consistent with the cytochemical data (Brachet,[141] Linet and Brachet[142]): a distinct drop in the PNA content of the nonnucleated halves is already apparent on the 3rd day after the operation. Ten days after enucleation, the nonnucleated parts contain, on the average, only one-third of their initial PNA.

There is no doubt that the removal of the nucleus is followed by a rapid and considerable drop of the PNA content in the cytoplasm: cytoplasmic PNA is unquestionably dependent on the nucleus for its prolonged maintenance. Similar conclusions can be drawn from the study of other cell types, e.g., maturing red blood cells: it is a well-known fact that reticulocytes represent an intermediary stage in hemopoiesis during which the cytoplasm still retains PNA although the nucleus has already been lost; but the reticulocytes are soon converted into mature erythrocytes, which have almost no PNA in their cytoplasm (Thorell,[69] Holloway and Ripley[143]).

Summing up, it might be said that there is at present good evidence that the cell nucleus plays a very important role in PNA and nucleotide metabolism: all the facts known, whether they refer to isotope studies, intracellular distribution of enzymes, or behavior of enucleated organisms, point in the same direction. On the other hand, whether nuclear PNA is a direct precursor of cytoplasmic PNA cannot yet be decided with certainty: although many facts are compatible with such a hypothesis, it is highly probable that, if such a transfer of nuclear PNA to the cytoplasm occurs, it is a more complex phenomenon than simple diffusion. Furthermore, it is

[139] G. H. Hogeboom and W. C. Schneider, *J. Biol. Chem.* **197,** 611 (1952).
[140] J. Brachet, *Experientia* **6,** 56 (1950).
[141] J. Brachet, *Symposia Soc. Exptl. Biol.* **6,** 173 (1952).
[142] N. Linet and J. Brachet, *Biochim. et Biophys. Acta* **7,** 606 (1951).
[143] B. W. Holloway and S. H. Ripley, *J. Biol. Chem.* **196,** 695 (1952).

very likely that nuclear PNA is not the *sole* precursor of cytoplasmic PNA, even though the presence of the nucleus is necessary for the prolonged maintenance of microsomes in enucleated organisms: recent experiments, made in this laboratory, show conclusively that the nonnucleated halves of certain unicellular organisms still incorporate orotic acid readily into their PNA fraction; but whether we are dealing with a true synthesis or a mere turnover is not yet known.

After this discussion on the role of the nucleus in PNA metabolism, we shall turn to another aspect of Caspersson's theory:[58,59,106] is the nucleus "a cell organelle organized especially for being the main center of the cell for the formation of proteins"?

(3) *The Role of the Nucleus in Protein Synthesis.* Isotope studies by Daly *et al.*[144] on incorporation of glycine-N^{15} in isolated nuclear and cytoplasmic proteins have shown that speed of incorporation varies considerably according to the tissue used; but incorporation always follows the same pattern, being high in both nuclear and cytoplasmic proteins in actively metabolizing tissues, such as liver or pancreas, and low in sperm or chicken erythrocytes. Among the nuclear proteins, the "residual proteins" show a more rapid uptake than the histones; the rate of incorporation in these residual proteins in liver is about the same as for mixed cytoplasmic protein, while it is lower in pancreas.

These results of Daly *et al.*[144] have been confirmed, for liver, by Smellie *et al.*,[127] working with formate-C^{14}, methionine-S^{35}, and glycine-N^{15}: the incorporation of all of these labeled substances into nuclear proteins has been found to be of the same magnitude as into the cytoplasmic proteins.

All these observations deal with mixed cytoplasmic proteins, i.e., the proteins of whole cytoplasm which has not been subjected to fractionation by differential centrifugation; results obtained on the incorporation of amino acids into the various cytoplasmic cell particles will be discussed later.

It is obvious from the existing data that the incorporation of labeled amino acids is no higher in the nucleus than it is in the cytoplasm, as might be expected if the nucleus were the main center of protein synthesis. It should, however, be emphasized that we know as yet nothing about the relative rates of incorporation of labeled amino acids into the nucleus and into the cytoplasm in tissues endowed with a high mitotic activity.

The capacity for nonnucleated cells such as reticulocytes to incorporate amino acids into their proteins is now a well-established fact: after the initial demonstration by London *et al.*[145] that reticulocytes, in contrast to mature erythrocytes, can incorporate labeled glycine into the heme moiety

[144] M. M. Daly, V. G. Allfrey, and A. E. Mirsky, *J. Gen. Physiol.* **36,** 173 (1952).
[145] I. M. London, D. Shemin, and D. Rittenberg, *J. Biol. Chem.* **183,** 749 (1950).

of hemoglobin, it has been conclusively proved by Borsook *et al.*[146,147] that radioactive leucine and other labeled acids are quickly incorporated into hemoglobin and other proteins. In the same laboratory, Holloway and Ripley[143] have shown that development of reticulocytosis is accompanied by a substantial increase in the PNA content, which is closely paralleled by the amount of radioactive leucine incorporated into the proteins. The authors point out that their results are compatible with the view that PNA is closely associated with amino acid incorporation into proteins; it might be added that they are not compatible with the view that the cell nucleus is the most important center of protein synthesis.

Slightly different results have, however, been reported by Koritz and Chantrenne,[148] who find that the maximal rate of incorporation of labeled glycine precedes the PNA maximum by 2 to 3 days; the PNA peak coincides with maximal content of the red blood cells in hemoglobin, dipeptidase, and carbonic anhydrase.

Nothing as yet is known about the capacity of nonnucleated *Amœba* halves to incorporate labeled amino acids into their proteins except the fact that autoradiographic techniques show that such an incorporation actually occurs 1 day after enucleation. But recent work by Brachet[16,141,149] and Urbani[150,151] has disclosed the interesting fact that different enzymes —therefore different proteins—behave in a very dissimilar manner in nonnucleated *Amœba* halves: while protease, amylase, and adenosinetriphosphatase remain completely unaffected by the removal of the nucleus, dipeptidase (as well as the total tyrosine-containing proteins: Brachet[152]) markedly decreases during the first 3 to 4 days; it then remains constant at a level which represents 50% of the amount present in the nucleated halves. A third group of enzymes, including acid phosphatase and esterase, remain constant for about 3 to 4 days and then drop sharply: they behave exactly as PNA.

These unexpected results show that the control exerted on protein maintenance by the nucleus differs according to the protein studied; it is very likely, but not yet definitely proved, that the enzymes which are unaffected by the removal of the nucleus are bound to large granules, comparable to the mitochondria, while those which behave like PNA are

[146] H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, *Federation Proc.* **10,** 18 (1951).

[147] H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, *J. Biol. Chem.* **196,** 669 (1952).

[148] S. B. Koritz and H. Chantrenne, *Arch. intern. physiol.* **60,** 549 (1952).

[149] J. Brachet, *Biochim. et Biophys. Acta* **9,** 221 (1952).

[150] E. Urbani, *Arch. intern. physiol.* **60,** 189 (1952).

[151] E. Urbani, *Biochim. et Biophys. Acta* **9,** 108 (1952).

[152] J. Brachet, *Nature* **168,** 205 (1951).

part of the microsomes. Dipeptidase seems to be, in the amœbæ, a soluble enzyme (see Holter,[153] Holter and Pollock[154] for a discussion of the intracellular localization of enzymes in amœbæ).

Another unicellular organism, the alga *Acetabularia mediterranea*, provides much more decisive proof that protein synthesis can go on for a long time in the absence of the nucleus: as shown already by Hämmerling's[155] beautiful experiments, a nonnucleated half can regenerate to a fairly large extent. Quantitative studies by Vanderhaeghe[156] have definitely shown that this regeneration is accompanied by an increase in dry weight and in protein; these synthetic processes go on for a fortnight at the same rate in the nucleated and nonnucleated halves; they then slow down and stop gradually in the enucleated half, which is, however, able to survive for several months. Experiments by Brachet and Chantrenne[157,158] with radioactive CO_2 have confirmed and extended these findings: incorporation of this precursor in the proteins is perfectly normal in nonnucleated halves for 2 weeks, when it begins to decrease. Nonnucleated halves are also able to incorporate radioactive glycine into their proteins (Brachet and Brygier[159]); it is interesting to note, in this respect, that while incorporation of CO_2 into the proteins occurs almost exclusively in the chloroplasts and is dependent on light supply, incorporation of glycine is more active in the microsomes than in the chloroplasts and is not affected by light: two different protein-synthesizing mechanisms, both largely independent of the presence of the nucleus, thus exist in this alga. Finally, it has been shown (Brachet and Chantrenne,[160] Chantrenne, Brachet, and Brygier[161]) that nonnucleated halves of *Acetabularia*, as well as nucleated ones, react to the addition of hydrogen peroxide to the medium by increased catalase activity, apparently due to adaptive synthesis: this capacity for increased catalase activity or production slows down in nonnucleated halves after 1 week, but it still exists in cytoplasmic fragments which have been removed from the nucleus for as long as 3 months.

These experiments clearly show that the nucleus exerts only a remote control on protein synthesis in this material: incorporation of amino acids

[153] H. Holter, *Advances in Enzymol.* **13,** 1 (1952).

[154] H. Holter and B. M. Pollock, *Compt. rend. trav. lab. Carlsberg, Sér. chim.* **28,** 221 (1952).

[155] J. Hämmerling, *Wilhelm Roux, Arch. Entwicklungsmech. Organ.* **131,** 1 (1934).

[156] F. Vanderhaeghe, *Arch. intern. physiol.* **60,** 190 (1952).

[157] J. Brachet and H. Chantrenne, *Nature* **168,** 950 (1951).

[158] J. Brachet and H. Chantrenne, *Arch. intern. physiol.* **60,** 547 (1952).

[159] J. Brachet and J. Brygier, *Arch. intern. physiol.* **61,** 248 (1953).

[160] J. Brachet and H. Chantrenne, *Arch. intern. physiol.* **61,** 246 (1953).

[161] H. Chantrenne, J. Brachet, and J. Brygier, *Arch. intern. physiol.* **61,** 419 (1953).

into proteins, and even synthesis of a specific enzyme protein, are fundamentally cytoplasmic processes.

It is interesting to note that very similar conclusions have been drawn by Bonner[162] in studies on the genetic control of enzyme formation in *Neurospora*: the facts that enzyme formation requires the presence of free amino acids, that the process rapidly leads to the formation of a specific enzyme, and that intermediates do not seem to accumulate, all point in the same direction: enzyme formation probably occurs in a cytoplasmic particle, and it is "the ability of such particles to replicate in an active form that is genetically controlled."

If we recall now that cytoplasmic PNA, which is certainly associated with protein synthesis, is mostly accumulated in the microsomes and that, in *Amœba*, these microsomes disappear after removal of the nucleus, it is tempting to identify them with Bonner's[162] hypothetical cytoplasmic particles. It is therefore to be expected that microsomes play some fundamental role in protein synthesis.

(4) *The Role of the Microsomes in Protein Synthesis.* In their first papers on the chemical composition of cytoplasmic particles, Brachet and Jeener[112] (see also Brachet[151]) pointed out that there is no reason to believe that PNA *alone* plays a part in protein synthesis: it is quite possible that the whole granule, the microsome, is the active agent.

They found some support for this hypothesis in the fact that particles obtained in the ultracentrifuge (which were in fact mixtures of mitochondria and microsomes) always contained an appreciable amount of the specific protein synthesized by each organ: this was found for trypsin and insulin in the pancreas, amylase in salivary glands, hemoglobin in red blood cells, and the melanophore-expanding hormone in the pituitary. More recently, McShan and Meyer[163] found more than 50% gonatropin in cytoplasmic granules. It should be added, however, that in none of these experiments was a separation made between microsomal and mitochondrial fractions.

More direct evidence comes from work done in other laboratories with labeled amino acids. Borsook *et al.*[164] were the first to report that incorporation in liver tissue is highest in the microsomes after intravenous injection of labeled amino acids (glycine, lysine, and leucine). The same result was also obtained by Hultin[165] using glycine-N^{15} in the chick: the uptake *in vivo* of the amino acid by the microsomal protein took place

[162] D. M. Bonner, *in* "Phosphorus Metabolism" (McElroy and Glass, eds.), Vol. 2, p. 153. Johns Hopkins Press, Baltimore, 1952.

[163] W. H. McShan and R. K. Meyer, *Proc. Soc. Exptl. Biol. Med.* **71**, 407 (1949).

[164] H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, *Federation Proc.* **9**, 154 (1950).

[165] T. Hultin, *Exptl. Cell Research* **1**, 376, 599 (1950).

more rapidly than in any other fraction, including mitochondria and nuclei. A high PNA concentration, characteristic of the microsomes, is therefore more important for protein synthesis than energy-producing systems present in the mitochondria according to Hultin.[165]

Later work by Tyner *et al.*[166,167] using glycine-C^{14}, by Keller[168] with labeled leucine, and by Lee *et al.*[169] using cystine-S^{35}, all in the rat, has entirely confirmed these early results; the same conclusion, i.e., that incorporation is greater in microsomal protein than in all other cellular fractions, is also reached by Smellie *et al.*,[127] who worked with formate-C^{14}, glycine-N^{15}, and methionine-S^{35}.

Work on homogenates *in vitro* also confirms the exceptional activity of the microsomal fraction in the incorporation of amino acids: such work has been done by Borsook *et al.*[170] and by Siekevitz,[171] who both emphasize, however, the importance of energy-yielding reactions for successful incorporation. For instance, Siekevitz[172] finds that incorporation of labeled alanine by liver homogenates requires the presence of both mitochondria and microsomes, the activity being greatest in the latter. The uptake is greatly increased by the addition of α-ketoglutarate to the system, while dinitrophenol and the hexokinase shunt are greatly inhibitory.

Experiments by Crampton and Haurowitz,[172] with antigens labeled with radio-iodine, have also shown that the antigen is very rapidly deposited in the microsomes: about 50% of the antigen deposited in the liver or spleen is found in this fraction a few minutes after injection. One hour later, most of the antigen is present in the mitochondria. This means, according to Haurowitz and Crampton,[173] that "since antibody synthesis is nothing but modified synthesis of normal plasma proteins, the synthesis of these proteins seems to take place in the cytoplasmic granules of liver, spleen and other tissues."

Another fact, which again is in agreement with the view that microsomes are especially concerned with protein synthesis, is the observation of Munro *et al.*[174] that, when rats are submitted to a protein-free diet, the PNA content of the liver decreases only for the microsomes: neither cell

[166] E. P. Tyner, C. H. Heidelberger, and G. A. LePage, *Federation Proc.* **11,** 300 (1952).
[167] E. P. Tyner, C. H. Heidelberger, and G. A. LePage, *Cancer Research* **12,** 158 (1952).
[168] E. B. Keller, *Federation Proc.* **10,** 106 (1951).
[169] N. D. Lee, J. T. Anderson, R. Miller, and R. H. Williams, *J. Biol. Chem.* **192,** 733 (1951).
[170] H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, *J. Biol. Chem.* **184,** 529 (1950).
[171] P. Siekevitz, *J. Biol. Chem.* **195,** 549 (1952).
[172] C. F. Crampton and F. Haurowitz, *Federation Proc.* **11,** 464 (1952).
[173] F. Haurowitz and C. F. Crampton, *Exptl. Cell Research* Suppl. 2, 45 (1952).
[174] T. W. Wikramanayake, F. C. Heagy, and H. N. Munro, *Biochim. et Biophys. Acta* **11,** 566 (1953).

sap PNA nor mitochondrial PNA are affected, while apparently the microsomes greatly decrease in number in animals on a protein-free diet.

While there is obviously a good deal of evidence favoring the opinion that cytoplasmic particles, microsomes in particular, are the major sites of protein synthesis in the cell, it would, however, be an exaggeration to believe that they are the *only* possible site of protein synthesis. Experimental work by Szafarz[174a] has shown that when the flagellate *Polytomella* is grown on labeled acetate-C^{14} as the sole carbon source, all cell particles have the same specific radioactivity in their proteins. If, when acetate has become the limiting factor for growth, an excess of ordinary acetate is added, brisk protein synthesis occurs, while the specific radioactivity of the various fractions of course decreases. The smaller the PNA-containing granules are, the faster is the drop in specific radioactivity. This might mean either that proteins of the smaller granules (microsomes) have the highest turnover and that these particles are of special importance in protein synthesis, or that their mass increases more rapidly than does that of the other fractions. That this second explanation is correct is shown by later work by Szafarz.[175a] When the flagellate is grown in continuous culture, so that all cell constituants grow at the same rate, the protein turnover is identical in all types of granules. Therefore, in the case of microorganisms kept continuously in the exponential phase of growth, microsomes cannot be the major source of cytoplasmic proteins; nor can these granules be obligate intermediates in protein synthesis. Such a conclusion is in agreement with the fact reported earlier that, in *Acetabularia*, microsomes or chloroplasts are the most important site of protein synthesis, depending on the precursor used (glycine or CO_2, respectively).

3. Biochemical Mechanisms of Protein Synthesis

The various aspects of this all-important biological and biochemical problem have been the object of many recent reviews (Northrop,[175] Lipmann,[176] Fruton,[177] Borsook,[178] Linderstrøm-Lang,[179] Haurowitz,[180,181] Chantrenne,[182] Brachet,[183] etc.). We shall discuss here only those aspects of protein synthesis which are linked together with PNA metabolism.

[174a] D. Szafarz, *Arch. internat. Physiol.* **60,** 196 (1952).
[174b] D. Szafarz, *Arch. internat. Physiol.* **61,** 269 (1953).
[175] J. H. Northrop, "Crystalline Enzymes," p. 232. Columbia Univ. Press, New York, 1948.
[176] F. Lipmann, *Federation Proc.* **8,** 597 (1949).
[177] J. S. Fruton, *Yale J. Biol. and Med.* **22,** 263 (1950).
[178] H. Borsook, *Physiol. Revs.* **30,** 206 (1950).
[179] K. Linderstrøm-Lang, *Proc. 6th Intern. Congr. Exptl. Cytol., Stockholm* p. 1 (1947).
[180] F. Haurowitz, *Quart. Rev. Biol.* **24,** 93 (1949).
[181] F. Haurowitz, *Biol. Revs.* **27,** 247 (1952).
[182] H. Chantrenne, *2nd Symposium Soc. Gen. Microbiol.* p. 1 (1953).
[183] J. Brachet, *Exposés ann. biochim. méd.* **12,** 1 (1950).

Briefly, three main theories for protein synthesis have been proposed: (a) protein synthesis results from a reversal of proteolysis; (b) protein synthesis involves the intervention of energy-rich (presumably phosphate) bonds; (c) protein synthesis occurs through a "template" mechanism. These three hypotheses will now be considered in turn.

a. Protein Synthesis by Reversal of Proteolysis

This is obviously the simplest possible hypothesis: proteases and peptidases, acting on amino acids, are the agents of protein synthesis.

It is a well-known fact that, when proteolytic enzymes act on mixtures of polypeptides, they can produce "plasteins," which are substances resembling proteins. However, the average molecular weight of the plasteins does not exceed 1,200 according to Ecker,[184] who concludes that they are little more than a complex mixture of peptides. Virtanen[185] believes that small peptides are synthesized by a phosphorylation process and large ones by reversal of proteolysis.

As has been pointed out by Linderstrøm-Lang,[186] there is no satisfactory evidence as yet that protein synthesis ever occurs when proteolytic enzymes are made to act on polypeptides; there have, however, recently been claims by Bresler and his co-workers[187-189] that, if a hydrolysate of a protein by a protease is submitted to very high pressures (e.g., 6,000 atm. for 18 hr. at 38°), resynthesis of the specific protein occurs to a certain extent. But it should be added that such a mechanism can hardly be physiological and, furthermore, that Bresler's claims for amylase activity have not been confirmed by Talwar *et al.*,[190] who found that high pressures inactivated the enzyme and who obtained no evidence of synthesis.

While it is doubtful whether proteolytic enzymes play an important part in protein synthesis, it is a well-established fact that they can catalyze peptide bond synthesis (Bergmann and Fruton[191]), as well as transpeptidation reactions (see Fruton[177] and Chantrenne[182] for a full discussion of these questions). But even though the transpeptidase activity of proteolytic enzymes is a factor which aids in the understanding of how amino acids

[184] P. G. Ecker, *J. Gen. Physiol.* **30,** 399 (1947).

[185] A. I. Virtanen, *Makromol. Chem.* **6,** 94 (1951).

[186] K. Linderstrøm-Lang, *Bull. soc. chim. biol.* **22,** 339 (1940).

[187] S. E. Bresler, M. V. Glikina, A. P. Konikov, N. A. Selezneva, and P. A. Finogenov, *Izvest. Akad. Nauk S. S. S. R., Ser. Fiz.* **13,** 396 (1949).

[188] S. E. Bresler, M. V. Glikina, and A. M. Tongur, *Doklady Akad. Nauk S. S. S. R.* **78,** 543 (1951).

[189] A. G. Pasynskiĭ and D. L. Talmud, *Doklady Akad. Nauk S. S. S. R.* **85,** 1361 (1952).

[190] G. P. Talwar, E. Barbu, J. Basset, and M. Macheboeuf, *Bull. soc. chim. biol.* **33,** 1793 (1951).

[191] M. Bergmann and J. S. Fruton, *Ann. N. Y. Acad. Sci.* **45,** 409 (1944).

might be selected and arranged in a polypeptide chain, it seems impossible to explain, on that basis alone, the synthesis of specific proteins (Chantrenne[182]).

It has been pointed out by Reiss,[192] as well as by Bergmann and Fruton,[191] that protein synthesis under the action of proteolytic enzymes would be favored if the reaction products were eliminated: this might occur in a polyphasic system, such as the cell, where adsorption or incorporation of the synthesized products on cytoplasmic particles (microsomes, for instance) could drive the reaction towards synthesis. Careful study of proteases distribution in the various cell particles would be necessary before such a possibility could be put to a test.

There are only a few indications that proteases might play a synthetic role in the cell: for instance, Schultz[193] found that fasting decreases simultaneously the number of cell particles in the liver and the amount of cathepsin II: this enzyme is apparently bound to cytoplasmic granules and it might play a role in protein synthesis. A similar relationship between catheptic activity and fixation of amino acids has been pointed out by Rothschild and Junqueira,[194] while Kritsman *et al.*[195] report that incorporation of labeled amino acids into serum proteins is increased by the addition of proteolytic enzymes. While the evidence for an intervention of proteases in protein synthesis and amino acid uptake by proteins is rather meager, it is by no means nonexistent, and further work in that direction is obviously needed before any conclusions can be drawn.

A much better understanding of the role of PNA in protein synthesis would be reached if a recent preliminary report by Binkley[196] could be confirmed that a dipeptidase, cysteinylglycinase, is of a pentose-polynucleotide nature. According to Binkley, the enzyme responsible for the hydrolysis of cysteinylglycine in pig kidney is identical with PNA.

The facts that this preparation is stable towards proteolytic enzymes and that it shows a typical nucleic acid absorption spectrum provide evidence for its PNA nature; however, as pointed out by Binkley[196] himself, the finding that the enzyme also resists ribonuclease digestion "is a source of some concern." Chantrenne's[197] observation that there is no parallelism between the sedimentation of cysteinylglycinase and of PNA when pigeon liver homogenates are centrifuged is a further reason for doubting the validity of Binkley's preliminary conclusions.

[192] P. Reiss, "L'action du potentiel d'oxydo-réduction du milieu sur l'activité des protéinases: hydrolyse et condensation." Clermont-Ferrand. 1942.
[193] J. Schultz, *J. Biol. Chem.* **178,** 451 (1949).
[194] H. A. Rothschild and L. C. U. Junqueira, *Arch. Biochem. and Biophys.* **34,** 453 (1951).
[195] M. G. Kritsman, A. S. Konikova, and T. D. Osipenko, *Biokhimiya* **17,** 488 (1952).
[196] F. Binkley, *Exptl. Cell Research* Suppl. 2, 145 (1952).
[197] H. Chantrenne, *Arch. intern. physiol.* **60,** 186 (1952).

Binkley's[196] paper ends with interesting, but highly speculative, hypotheses on the mechanism of protein synthesis; the first stage is ascribed to PNA, acting as dipeptidase for the synthesis of dipeptides. Such a suggestion would be in keeping with Linderstrøm-Lang's[179,186] observation that dipeptidases are always found in large amounts in active protein-synthesizing cells, as well as with Fruton's[177] and Chantrenne's[182] views on transpeptidation reactions in protein synthesis.

b. Energy-rich Bonds in PNA Synthesis

The necessity for an energy supply for peptide synthesis has been emphasized repeatedly, and Borsook[178] has conclusively demonstrated that incorporation of labeled amino acids into proteins requires energy: the process is stopped, or markedly reduced, by anaerobiosis or addition of cyanide, azide, dinitrophenol, etc. In Siekevitz's[171] opinion, this uptake of labeled amino acids into proteins is more closely linked to phosphorylation than to oxidation.

Such a conclusion is in keeping with Lipmann's[176] and Chantrenne's[182] views on the mechanism of peptide bond synthesis and with the possibility, which has been successfully tested experimentally by Chantrenne,[182] that phosphorylated amino acids might be intermediaries in peptide synthesis.

It should be pointed out, however, that these problems relate to peptide synthesis only, and protein synthesis might be a very different process. It should also be noted that Borsook's[178] experimental findings might have an alternative explanation. It has been recently reported by Simpson[198] that not only the uptake, but also the liberation, of amino acids from liver slices is markedly inhibited by anaerobiosis, cyanide, dinitrophenol, etc.; it might thus well be that liberation of amino acids from intracellular proteins also requires energy. If such were the case, proteolysis might occur by two different mechanisms, one of which required energy. If this were so, one of the reasons for doubting the intervention of proteases in protein synthesis would lose much of its weight.

The suggestion has been made by Spiegelman[199] that phosphorylated PNA might be a specific phosphate and energy donor for protein synthesis; later work by Spiegelman and Kamen[200] has shown, however, that what had been taken for phosphorylated PNA probably is a mixture of ordinary PNA and metaphosphate. The whole question of the frequent association of metaphosphate and PNA is, however, still in a very confused state, and much more work will be required before the existence of phosphorylated PNA can be accepted or rejected.

[198] M. V. Simpson, *J. Biol. Chem.* **201,** 143 (1953).

[199] S. Spiegelman, *Cold Spring Harbor Symposia Quant. Biol.* **11,** 256 (1946).

[200] S. Spiegelman and M. D. Kamen, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 211 (1947).

There are, nevertheless, a few indications in favor of the existence of phosphorylated PNA. It has been recently reported by Bressler and Nidzyan[201]—and confirmed in this laboratory—that if ATP labeled with P^{32} is mixed with PNA in the presence of a liver homogenate, part of the radioactive P is transferred to PNA or, at any rate, to a substance absorbing ultraviolet light and resembling PNA chromatographically.

In a paper dealing with the use of firefly luminescence for ATP estimation, Strehler and Totter[202] incidentally report that some purified PNA's have been found to have "ATP" activity; this activity does not disappear after treatment with ribonuclease. It is interesting to note that, according to Strehler and Totter,[202] PNA's from growing yeast cultures have "ATP" activity, while samples from dormant cultures do not.

While these findings provide interesting hints that phosphorylation of PNA might be a step in protein synthesis, it must be admitted that the evidence is still too meager to allow any definite conclusion. At the present time, we are still unable to decide whether peptide synthesis is due to reversal of proteolysis or to intermediate phosphorylation reactions, or to both; and the difficulties of explaining protein specificity are even more formidable.

c. The "Template" Hypothesis

For the biologist, especially the geneticist and the immunologist, the major problem to be solved is the mechanism of *specific* protein synthesis rather than the nature of the energy-yielding reactions. The problem here is merely at the stage of ingenious hypotheses, the major one, for which there is no satisfactory substitute so far, being the so-called template hypothesis, which postulates the existence of a model (template) under the influence of which the building blocks, whether they are amino acids or peptides, are arranged in the right order. The template would act as a mold forming a counterpart to the protein to be formed. It is tempting to suppose, as many have already done (Friedrich-Freksa,[203] Rondoni,[204] Haurowitz,[173,180,181] and Caldwell and Hinshelwood[205]), that it is PNA which represents the counterpart to the protein.

It would be fruitless to go into details of all these theories, but those of Haurowitz[173,180,181] and of Caldwell and Hinshelwood[205] deserve further mention. Haurowitz[173] believes that protein synthesis takes part in two successive steps. The first stage is the combination of amino acids to form a definite species-specific peptide pattern; in the second phase, the peptide

[201] S. E. Bresler and E. I. Nidzyan, *Doklady Akad. Nauk S. S. S. R.* **75,** 79 (1950).
[202] B. L. Strehler and J. R. Totter, *Arch. Biochem. and Biophys.* **40,** 28 (1952).
[203] H. Friedrich-Freksa, *Naturwissenschaften* **28,** 376 (1940).
[204] P. Rondoni, *Enzymologia* **9,** 380 (1940).
[205] P. C. Caldwell and Sir Cyril Hinshelwood, *J. Chem. Soc.* **1950,** 3156.

folds to form a three-dimensional globular protein molecule. The main function of PNA would be to maintain the protein template in the expanded state of a film.

Caldwell and Hinshelwood[205] point out many analogies between autosynthesis and the growth of crystals. They believe that nucleic acids, by a process akin to crystallization, impose a definite order on the arrangement of the amino acids. A converse mechanism would control synthesis of specific nucleic acids by the newly built protein. Such a theory requires the presence in the cell of a large number of self-duplicating nucleoproteins, of which the protein part would have specific enzymic activity. Furthermore, if we admit that PNA acts as an organizer or template for protein synthesis, it follows that increased PNA will lead to increase in protein production.

While there is no evidence to prove conclusively the existence of a template mechanism, many experimental results fit in well with this hypothesis. These include Gale and Folkes'[93] observation that certain antibiotics, as already found by Levy *et al.*[94] and Miura *et al.*[95,96] for cobaltous ions and usnic acid, inhibit protein synthesis while the rate of PNA formation is increased. The antibiotics presumably act on the splitting off of the nucleoprotein complex. As Hammarsten[206] points out, these experiments emphasize that protein synthesis is secondary to PNA synthesis, and they suggest that nucleoprotein synthesis not only precedes protein synthesis but is even necessary for the latter. It has been demonstrated that in pancreatic tissue stimulated to enzyme production by pilocarpine (Hokin,[207] De Deken-Grenson,[208,209]) and in the secreting oviduct of laying hens (Grenson[210]), the synthesis of proteins is not linked to the rate of uptake of P^{32} into PNA. These observations are in better agreement with the template hypothesis than with any other. As Hokin[207] points out, it would seem that PNA plays a part during the rearrangement and movement of enzymes during secretion. Nucleoproteins or PNA might act as a specific framework onto which enzyme systems could be organized and which could direct the synthesis of more PNA. Such a view is consistent with the results of Daly and Mirsky,[211] indicating that the total protein content of the pancreas remains constant during the cycle of secretion and synthesis: when enzyme secretion takes place, rapid synthesis of a precursor protein would occur, which would be followed by gradual transformation into the characteristic pancreatic enzymes.

[206] E. Hammarsten, *Ciba Conf. on Isotopes in Biochem., London* p. 203 (1951).
[207] L. E. Hokin, *Biochim. et Biophys. Acta* **8**, 225 (1952).
[208] M. De Deken-Grenson, *Biochim. et Biophys. Acta* **10**, 480 (1953).
[209] M. De Deken-Grenson, *Biochim. et Biophys. Acta* **12**, 560, 1953.
[210] M. Grenson, *Biochim. et Biophys. Acta* **9**, 102 (1952).
[211] M. M. Daly and A. E. Mirsky, *J. Gen. Physiol.* **36**, 243 (1952).

Finally, the fact mentioned earlier in this report that incorporation of thiouracil into the PNA of virus-diseased plants decreases markedly and simultaneously the turnover rates of normal and virus proteins, as well as those of the purines and phosphorus in normal and virus PNA (Jeener[102]), is obviously in excellent agreement with the template hypothesis; alterations in the chemical constitution of the PNA model or organizer should lead to disturbances in protein synthesis.

It is possible that the microsomes, which are rich in PNA and incorporate amino acids very actively, contain the templates on which the proteins are synthesized; but whether the building blocks for protein synthesis are free amino acids or intermediates such as polypeptides, is still open to discussion. Genetic evidence suggests that enzyme synthesis requires the presence of free amino acids; if intermediates exist, they do not accumulate (Bonner[162]). Similar conclusions can be drawn from work by Halverson and Spiegelman[212] on the inhibition of enzyme formation by amino acid analogues: "free amino acid metabolism constitutes a major component of the enzymes synthesizing mechanism." Similar implications arise from Hokin's[213] work, which shows that the synthesis of amylase by pancreas slices is markedly increased by the addition of the essential amino acids present in the enzyme molecule.

On the other hand, isotope experiments by Anfinsen and Steinberg[214-216] on the syntheses of ovalbumin in minces of surviving oviduct and of ribonuclease in pancreas slices show that the specific activities of the labeled amino acid (alanine for ovalbumin, phenylalanine for ribonuclease) vary in different parts of the molecule; these findings strongly suggest that peptides are intermediates in protein synthesis, a conclusion also accepted by Campbell and Work[217] in studies on the biosynthesis of milk proteins.

Recent work by Peters[218] and by Koritz and Chantrenne[148] has further shown that incorporation of labeled amino acids precedes the appearance of specific labeled proteins: the conversion of amino acids into protein requires time, but, as Peters points out, the results are compatible both with the intermediate or peptide theory and with the template hypothesis.

But, whatever the real mechanism may be, there is no doubt that protein synthesis *in vitro*, as well as the uptake of amino acids, requires energy: whether this energy is required for initial peptide synthesis only or for the building up of complete specific proteins on a template as well is not known.

[212] H. O. Halvorson and S. Spiegelman, *J. Bacteriol.* **64,** 207 (1952).
[213] L. E. Hokin, *Biochem. J.* **50,** 216 (1951).
[214] C. B. Anfinsen and D. Steinberg, *J. Biol. Chem.* **189,** 739 (1951).
[215] D. Steinberg and C. B. Anfinsen, *Federation Proc.* **11,** 292 (1952).
[216] D. Steinberg and C. B. Anfinsen, *J. Biol. Chem.* **199,** 25 (1952).
[217] P. N. Campbell and T. S. Work, *Biochem. J.* **52,** 217 (1952).
[218] T. Peters, Jr., *J. Biol. Chem.* **200,** 461 (1953).

If phosphorylated forms of PNA really exist, they might raise the energy level of activated amino acids on the surface of the cytoplasmic template (microsomes). But other possibilities for energy-rich bonds within the microsomes still exist and should not be forgotten. It is known, for instance, as a result of Brachet and Jeener's[112] early work, that cytoplasmic particles give strong reactions for —SH groups; and it is now a well-established fact that —S $\sim$ PO_3H_2 bonds are energy rich (Lipmann,[219] Walsh[220]). The possible significance of such bonds is strengthened by Peterson and Greenberg's[221] observation that a considerable proportion of the radioactivity of mitochondria incubated with labeled amino acids is removed by interaction with mercaptoethanol. Such —S $\sim$ PO_3H_2 groups might represent a counterpart in the microsomes of the phosphoarginine groups which have been postulated by Stern[222] to account for gene synthesis in chromosomes. Still another possibility lies in the fact, recently discovered by Wieland and Schneider[223] and by Stadtman and White,[224] that acetyl derivatives of imidazole are energy-rich compounds: these findings suggest that the imidazole ring of histidine might take part in the transfer or activation of acyl groups, but similar conditions might also prevail in the case of the purines.

In summary, it may be said that the work of recent years has considerably strengthened the hypothesis that PNA plays a role in protein synthesis; the evidence comes from cytochemical observations, studies on plant virus multiplication, analysis of morphogenesis, biochemical experiments on the uptake of labeled amino acids, etc. But, however convincing, this evidence still remains circumstantial: what is required now is the development of new methods for the isolation of "native" PNA's and decisive experiments for testing the biochemical and biological properties of these substances. Such experiments might very well show that PNA, like DNA in the phage and transforming principle systems, plays an important genetic role and that it acts as a catalyst in protein synthesis.

IV. Addendum

So many papers have been devoted, during the past few months, to the various aspects of the biological role of PNA that the most important only can be briefly summarized here.

In the field of plant viruses, valuable contributions have been made by

[219] F. Lipmann, *Bacteriol. Revs.* **17,** 1 (1953).
[220] E. O. F. Walsh, *Nature* **169,** 546 (1952).
[221] E. A. Peterson and D. M. Greenberg, *J. Biol. Chem.* **194,** 359 (1952).
[222] K. G. Stern, *Yale J. Biol. and Med.* **19,** 937 (1947).
[223] T. Wieland and G. Schneider, *Ann.* **580,** 159 (1953).
[224] E. R. Stadtman and F. H. White, *J. Am. Chem. Soc.* **75,** 2022 (1953).

Matthews,[225] who found that 8-azaguanine is incorporated in TMV virus PNA, with a simultaneous loss of infectious power, in agreement with the results with thiouracil obtained by Jeener and Rosseels.[10] These findings have been extended by Lesnitzki, Matthews, and Smith[226] to various animal cells and bacteria. Jeener, Lemoine and Lavand'homme's[227] experiments on the production of a nonvirulent protein antigenically related to the virus, have also been confirmed and extended by Commoner *et al.*[228]

Regarding the role of PNA in embryonic development, Osawa and Hayashi[229] and Takata[230] have confirmed, by direct chemical analysis, the cytochemical observations of Brachet[15] on the PNA distribution in oocytes, as well as in gastrulæ and in neurulæ. Of special interest is the finding by Niu and Twitty[231] that explanted chordomesoderm produces in the medium a neuralizing agent: they have found that isolated ectoderm differentiates nerve fibers and pigment cells when cultivated in the saline medium in which chordomesoderm has been explanted for a few days. This medium shows a strong ultraviolet absorption typical of nucleic acids. A comparable finding is that of Levi-Montalcini and Hamburger,[232] who discovered a diffusible agent from mouse sarcoma that produces hyperplasia of sympathetic ganglia. According to a recent report by Cohen, Levi-Montalcini and Hamburger,[233] this agent is accumulated in the microsomes, is precipitated by streptomycin, and shows a typical nucleic acid ultraviolet spectrum. There is little doubt that, in Hamburger's case, the active agent is a PNA. Favorable effects of PNA on the regeneration of Planarians have been reported by Brønsted and Brønsted,[234] while nucleoproteins (but not PNA alone) have been found to stimulate growth in tissue cultures (Kutsky,[235] Maganini *et al*[236]).

While these findings are all in favor of an important role of PNA in morphogenesis and cell multiplication, Elson and Chargaff[237] have, how-

[225] R. E. F. Matthews, *J. Gen. Microbiol.* **10,** 521 (1954).
[226] I. Lasnitzki, R. E. F. Matthews, and J. D. Smith, *Nature* **173,** 346 (1954).
[227] R. Jeener, P. Lemoine, and C. Lavand'homme, *Biochim. et Biophys. Acta* **14,** 321–334 (1954).
[228] B. Commoner, Y. Yamada, S. D. Rodenberg, Tung-Yue Wang, and E. Basler, Jr., *Science* **118,** 529 (1953).
[229] S. Osawa and Y. Hayashi, *Science* **118,** 84 (1953).
[230] A. Takata, *Biol. Bull.* **105,** 348 (1953).
[231] M. C. Niu and V. C. Twitty, *Proc. Natl. Acad. Sci. U. S.* **39,** 985 (1953).
[232] R. Levi-Montalcini and V. Hamburger, *J. Exptl. Zool.* **123,** 233 (1953).
[233] S. S. Cohen, R. Levi-Montalcini, and V. Hamburger, *Proc. Natl. Acad. Sci. U. S.* **40,** 1014 (1954).
[234] A. Brøndsted and H. V. Brøndsted, *J. Embryol. Exptl. Morphol.* **1,** 49 (1953).
[235] R. J. Kutsky, *Proc. Soc. Exptl. Biol. Med.* **83,** 390 (1953).
[236] H. Maganini, A. W. Schweitzer, and G. M. Hass, *Federation Proc.* **12,** 453 (1953).
[237] D. Elson, T. Gustafson, and E. Chargaff, *J. Biol. Chem.* **209,** 285 (1954).

ever, reported that PNA remains constant both in amount and in chemical composition during development of sea urchin eggs, even if abnormalities are produced by lithium treatment.

Papers by Anderson and Åquist,[238] Bennett,[239] Tyner *et al.*,[240] and Hammarsten *et al.*[241] on the incorporation of various precursors in the PNA and in the proteins of homogenates confirm, on the whole, the results obtained by Smellie *et al.*[127] which have been discussed at some length in the preceding article. Regarding the role of the nucleus in PNA synthesis or turnover, recent work with autoradiographic techniques (A. Ficq,[242] J. Taylor[243] shows clearly that the nucleolus plays a leading part: incorporation of P^{32} or radioactive glycine is exceptionally rapid in the nucleolar PNA, a conclusion also drawn by Stich and Hämmerling,[244] who studied the radioactivity of the nucleolus dissected out of giant *Acetabularia* algæ treated with P^{32}. In the starfish oocytes studied by A. Ficq,[242] incorporation of glycine in the nucleolar proteins is also usually high and fast. While these autoradiographic results lend support to Caspersson's theory, it has been found by Brachet and Szafarz[245] that PNA turnover, followed with labeled orotic acid, is not seriously effected by the removal of the nucleus in *Acetabularia*. Such a finding is somewhat difficult to reconcile with the suggestion that cytoplasmic PNA originates within the nucleus (Caspersson,[58] Jeener and Szafarz[124]). This hypothesis, indeed, is steadily losing ground: Barnum, Huseby, and Vermund,[246] on the basis of new experimental data and extensive calculations, come to the definite conclusion that their results are not consistent with the assumption that nuclear PNA is the precursor of any fraction of cytoplasmic PNA. The same opinion is shared by Moldave and Heidelberger,[247] who found that the PNA's of the nuclei, mitochondria, microsomes, and supernatant are all different from each other in both the composition of bases and the nucleotides sequence.

Coming now to the question of the part played by the *nucleus in protein synthesis*, we have seen earlier that biochemical experiments on homogenates have so far failed to show a larger incorporation into the isolated nuclei than into the mixed cytoplasmic proteins. In contrast to these findings,

[238] E. P. Anderson and S. E. G. Åqvist, *J. Biol. Chem.* **202,** 513 (1953).
[239] E. L. Bennett, *Biochim. et Biophys. Acta* **11,** 487 (1953).
[240] E. P. Tyner, C. Heidelberger, and G. A. LePage, *Cancer Research* **13,** 186 (1953).
[241] E. Hammarsten, B. Thorell, S. Åquist, N. Eliasson, and L. Åkerman, *Exptl. Cell Research* **5,** 404 (1953).
[242] A. Ficq, *Experientia* **9,** 377 (1953).
[243] J. H. Taylor, *Science* **118,** 555 (1953).
[244] H. Stich and J. Hämmerling, *Z. Naturforsch.* **8b,** 329 (1953).
[245] J. Brachet and D. Szafarz, *Biochim. et Biophys. Acta* **12,** 588 (1953).
[246] C. P. Barnum, R. A. Huseby, and H. Vermund, *Cancer Research* **13,** 880 (1953).
[247] K. Moldave and C. Heidelberger, *J. Am. Chem. Soc.* **76,** 679 (1954).

autoradiographic observations by Ficq[242] and by Ficq and Errera[248] have shown, in the liver, a much higher incorporation of radioactive glycine into the nuclear proteins than into the cytoplasmic ones; in the starfish oocyte (Ficq[242]), incorporation of the labeled amino acid into the nucleolar proteins is unusually high and fast. The obvious discrepancy between the biochemical and the autoradiographic results, in the case of the liver, might well be due to the fact that the active nuclear proteins are apparently very soluble: treatment of the sections with media generally used for the isolation of nuclei in homogenates (citric acid, sucrose, etc.) markedly decreases the radioactivity of the nuclei (Ficq and Errera[248]). Experiments on the incorporation of labeled amino acids with the proteins of nuclei isolated in non-aqueous media should throw additional light on the question. If the autoradiographic observations are correct, the situation regarding the incorporation of labeled precursors into the nucleus and the cytoplasm is the same for PNA and proteins: in both cases, incorporation is faster in the nucleus than in the cytoplasm, but appreciable synthesis nevertheless occurs for a considerable time in non-nucleated cytoplasm. That protein synthesis can go on in the absence of the nucleus has again been confirmed with reticulocytes by recent experiments of Nizet and Lambert[249] and from Koritz and Chantrenne.[250] It is interesting, in this respect, to mention that autoradiographic observations by Gavosto and Rechenmann[251] have conclusively shown that decrease in PNA content and decrease in glycine incorporation into the proteins run perfectly parallel during the maturation of the reticulocytes.

The lively and interesting discussion which has gone on regarding the template theory of protein synthesis can only be mentioned here (Campbell and Work,[252,253] Dounce,[254,255] Gale[256] Dalgliesh[257]); new evidence has been brought forward in favor of the view that protein synthesis occurs at the expense of free amino acids (Halvorson and Spiegelman[258,259]); but Anfinsen and Flavin[260] have extended to ribonuclease their earlier findings on ovalbumin, indicating rather a stepwise synthesis of proteins. In connec-

[248] A. Ficq and M. Errera, *Biochim. et Biophys. Acta* **16,** 45 (1955).
[249] A. Nizet and S. Lambert, *Bull. soc. chim. biol.* **35,** 771 (1953).
[250] S. B. Koritz and H. Chantrenne, *Biochim. et Biophys. Acta* **13,** 209 (1954).
[251] F. Gavosto and R. Rechenmann, *Biochim. et Biophys. Acta* **13,** 583 (1954).
[252] P. N. Campbell and T. S. Work, *Nature* **171,** 997 (1953).
[253] P. N. Campbell and T. S. Work, *Nature* **172,** 541 (1953).
[254] A. L. Dounce, *Enzymologia* **15,** 251 (1952).
[255] A. L. Dounce, *Nature* **172,** 541 (1953).
[256] E. F. Gale, *Advances in Protein Chem.* **8,** 285 (1953).
[257] S. C. Dalgliesh, *Nature* **171,** 1027 (1953).
[258] H. O. Halvorson and S. Spiegelman, *J. Bacteriol.* **65,** 496 (1953).
[259] H. O. Halvorson and S. Spiegelman, *J. Bacteriol.* **65,** 601 (1953).
[260] C. B. Anfinsen and M. Flavin, *Federation Proc.* **12,** 170 (1953).

tion with the role played by PNA in protein synthesis, Dounce and Kay[261] have published the very interesting, but as yet preliminary, report that PNA can be phosphorylated by the ATP-myokinase system.

However, the most important—and to the author of this review the most gratifying—discoveries in the field of the biological activities of PNA come from a series of recent papers which report that ribonuclease has been used as an analytical tool for biochemical experiments. The most complete and interesting results so far reported are undoubtedly those of Gale and Folkes.[262,263] Working on disintegrated *Staphylococci* which no longer respire, nor multiply, they found that these "cells" can still incorporate amino acids into their proteins, provided ATP and hexosediphosphate are present in the medium. Removal of the nucleic acids by nucleases or NaCl treatment strongly reduces this incorporation; addition of the nucleic acids fraction restores to a large extent the capacity to incorporate several of the amino acids into the proteins. Going a step further, Gale and Folkes[263] studied the effects of removal and addition of the nucleic acids on the actual synthesis of several enzymes including catalase and the adaptive synthesis of β-galactosidase. Here, again definite results were obtained in the case of the disrupted cells, the nucleic acids of which had been removed. Addition of bacterial PNA was most active in enhancing the synthesis of catalase while DNA, especially if isolated from adapted cells, proved to be the best for the stimulation of the synthesis of the adaptive β-galactosidase. Gale and Folkes[263] conclude, somewhat along the line of Caspersson's ideas, that DNA acts as an organizer for the synthesis of specific PNA's, which somehow catalyze protein synthesis.

Similar but less complete results have also been reported by Lester,[264] who found that *Micrococcus lysodeikticus* when lysed with lysozyme in the presence of sucrose, is still able to incorporate labeled amino acids into its proteins. Here again, ribonuclease exerts a powerful inhibitory effect on the phenomenon. Deoxyribonuclease, on the contrary, stimulates the incorporation.

It has been amply demonstrated, especially by Borsook and his school, that the incorporation of amino acids into proteins is an energy requiring process; the possibility is thus open that, in the experiments of Lester,[264] ribonuclease somehow inhibits the oxidative mechanisms of the cell. A recent investigation by Beljanski[265] in this laboratory has ruled out this possible objection by showing that ribonuclease has no effect on the respiration of lysed *Micrococcus lysodeikticus* cells. Similarly, it has been reported re-

[261] A. L. Dounce and E. R. M. Kay, *Proc. Soc. Exptl. Biol. Med.* **83,** 321 (1953).
[262] E. F. Gale and J. P. Folkes, *Nature* **173,** 1223 (1954).
[263] E. F. Gale and J. P. Folkes, *Nature* **173,** 1223 (1954).
[264] R. L. Lester, *J. Am. Chem. Soc.* **75,** 5448 (1953).
[265] M. Beljanski, *Biochim. et Biophys. Acta* **15,** 425 (1954).

cently by Pavlova[266] that protease-free ribonuclease exerts no influence on oxidative phosphorylations in homogenates.

The obvious next step, under such circumstances, was to study the possible effects of ribonuclease on the incorporation of amino acids into the proteins of homogenates. As we already know from Siekevitz's work, such an incorporation occurs mostly in the microsomes and requires the presence of an ATP generating system. Recent experiments of Allfrey *et al.*,[267] although still of a preliminary nature, have shown that the integrity of PNA is necessary for the incorporation of amino acids in the proteins of the microsomes: adding ribonuclease to the system again strongly inhibits the incorporation process. Similar observations have, still more recently, been reported by Zacmenik and Keller,[268] who found that the incorporation of amino acids into the proteins proceeds very well in anaerobiosis, provided an adequate ATP generating system is present. All that is needed for the reaction to proceed are the microsomes, a nondialyzable soluble fraction, and the ATP generating system. The incorporation mainly occurs in the proteins of the microsomes and, again, ribonuclease acts as an inhibitor. This simplified biochemical system of Zacmenik and Keller[268] will certainly be helpful in the understanding of the biochemical mechanisms of protein synthesis. Two factors of the machinery are already identified, ATP and PNA.

As repeatedly emphasized earlier, the microsomes are especially rich in PNA. This is the reason why the present writer suggested, some 10 years ago, that they must be very important in protein synthesis. Impressive experimental support for this opinion has been recently brought forward by Allfrey *et al.*[267] who found that there is a correlation between the PNA content of the microsome fraction pellet and the rate of protein synthesis in a tissue and that, in the pancreas, the pellet protein serves as a precursor material in the synthesis of the secretory proteins. Similar results have been obtained and the same conclusions have been drawn by Oota and Osawa,[269] working on plant material. It thus seems to be a general property of all living organisms that microsomes, because of their high PNA content, play an exceedingly important role in protein synthesis; very similar conclusions, based mainly on recent cytochemical evidence, have also been reached by Pollister[270] in an excellent review of the problem.

The importance of PNA in protein synthesis in disrupted bacteria and in microsomes seems thus to have been established beyond doubt; but does

[266] M. V. Pavlova, *Doklady Akad. Nauk S. S. S. R.* **92,** 641 (1953).
[267] V. G. Allfrey, M. M. Daly, and A. E. Mirsky, *J. Gen. Physiol.* **37,** 157 (1953).
[268] P. C. Zamecnik and E. B. Keller, *J. Biol. Chem.* **209,** 337 (1954).
[269] Y. Oota and S. Osawa, *Biochim. et Biophys. Acta* **15,** 162 (1954).
[270] Pollister, A. W., "Dynamics of Growth Processes," pp. 33–169. Princeton Univ. Press, Princeton, 1954.

PNA play the same essential role in protein synthesis by *living* cells? This is the question we have recently been trying to answer (Brachet[271]) using again ribonuclease as an analytical tool. Experiments by Kaufmann and Das[272] have shown that ribonuclease penetrates into living root-tip cells, where the enzyme induces various mitotic abnormalities. Ribonuclease, as shown by our co-worker Ledoux,[273,274] also inhibits quickly and powerfully the cleavage of Amphibian eggs.

The main results obtained from onion roots (Brachet[271]) are as follows: ribonuclease quickly inhibits the incorporation of labeled amino acids into the proteins of the living cells without affecting at all the respiration of the root tips. The enzyme first stimulates, then inhibits, the incorporation of labeled adenine into PNA. The incorporation of labeled adenine in DNA is, on the contrary, immediately inhibited. The total amount of PNA drops by some 20 % in the enzyme treated roots, while free nucleotides accumulate. Phosphorylations seem to be affected by ribonuclease, since its action leads to a drop in the inorganic phosphate and an increase in the ATP contents.

These results clearly show that PNA also plays a very important part in the incorporation of labelled amino acids into the proteins of living, normally respiring cells. The fact that a large proportion of PNA remains intact speaks rather in favor of a template mechanism, in which partially degraded PNA would prove useless. The simultaneous accumulation of ATP points toward an imperfect utilization of this substance and would fit in with Dounce's[254] hypothesis of a phosphorylated PNA as the active template.

It is obvious, from the unusual length of this addendum, that impressive progress has been made during the past few months; it is to be expected that many more spectacular advances will be made in the near future. We have now the possibility of studying the role of PNA in living cells as well as in relatively simple systems. The problem of the role of PNA in protein synthesis is no longer cytochemical; it has finally become a biochemical one. Very soon, PNA will cease to be a mysterious "*deus ex machina*" and we will really begin to understand how it works.

The author wishes to thank Professor J. N. Davidson, Professor R. Jeener, and Professor H. Chantrenne for placing manuscripts of papers in press at his disposal and for invaluable help in improving the text of the present review.

[271] J. Brachet, *Nature* **174,** 876 (1954).
[272] B. P. Kaufmann and N. K. Das, *Carnegie Inst. Wash. Year Book* **52,** 238 (1953).
[273] L. Ledoux, *Biochim. et Biophys. Acta* **15,** 143 (1954).
[274] L. Ledoux, J. Le Clerc, and F. Vanderhaeghe, *Nature* **174,** 793 (1954).

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