IONIZING RADIATIONS AND CELL METABOLISM

Ciba Foundation Symposia

General Volumes:

Visceral Circulation – – – – –

Mammalian Germ Cells – – – –

Preservation and Transplantation of Normal Tissues – – – – – – –

Hypertension: Humoral and Neurogenic Factors – – – – – – –

Leukæmia Research – – – – –

Chemistry and Biology of Pteridines – –

Experimental Tuberculosis – – – –

Porphyrin Biosynthesis and Metabolism –

Histamine – – – – – – –

Extrasensory Perception – – – –

Bone Structure and Metabolism – – –

Paper Electrophoresis – – – – –

The Nature of Viruses – – – – –

Chemistry and Biology of Purines – – –

A leaflet giving fuller details of these volumes, also of the Ciba Foundation Colloquia on Endocrinology and Colloquia on Ageing, is available from the Publishers.

CIBA FOUNDATION SYMPOSIUM
ON
IONIZING RADIATIONS
AND
CELL METABOLISM

Editors for the Ciba Foundation

G. E. W. WOLSTENHOLME, *O.B.E.*, M.A., M.B., B.Ch.

and

CECILIA M. O'CONNOR, B.Sc.

With 48 Illustrations

LITTLE, BROWN AND COMPANY
BOSTON

Published in London by
J. & A. Churchill Ltd.
104 *Gloucester Place, W.*1

First published 1956

Printed in Great Britain

PREFACE

THE Ciba Foundation, London, is an educational and scientific charity founded by a Trust Deed made in 1947. Its distinguished Trustees, who are wholly responsible for its administration, are The Rt. Hon. Lord Adrian, O.M., F.R.S.; The Rt. Hon. Lord Beveridge, K.C.B., F.B.A.; Sir Russell Brain, Bt.; The Hon. Sir George Lloyd-Jacob, and Mr. Raymond Needham, Q.C. The financial support is provided by the world-wide chemical and pharmaceutical firm which has its headquarters in Basle, Switzerland.

The Ciba Foundation forms an international centre where workers active in medical and chemical research are encouraged to meet informally to exchange ideas and information. It was opened by Sir Henry Dale, O.M., F.R.S., in June 1949.

In the first seven years, in addition to many part-day discussions, there have been 40 small international symposia, each lasting two to four days and attended by from twenty-five to thirty outstanding workers from many countries. Other symposia are planned at the rate of five or six a year.

The informality and intimacy of these meetings have permitted discussion of current and incomplete research and stimulated lively speculation and argument. They have also been the occasion for reference to much published and unpublished work throughout the world.

The reader will probably be well aware that there have been many conferences, national, international and inter-disciplinary, in recent years on the effects and hazards of radiation. This is partly due to rapid progress and expansion in this field of research, and partly to a quickening interest in the significance of the work shown by other scientists and by laymen. Most of these conferences have been on a fairly large scale, valuable for exchanges of information but usually affording little opportunity, except privately, for thorough discussion.

Dr. A. Hollaender and Professor A. Haddow made these points when approaching the Director late in 1954 with a request that a symposium on the Influence of Ionizing Radiations on Cell Metabolism should be included in the Ciba Foundation's programme. The Director readily agreed, subject to receiving their expert advice on its organization, in which they were later most helpfully joined by Professor J. A. V. Butler and Dr. L. H. Gray.

The symposium, which was realized in March 1956, and which was held under the skilful and kindly chairmanship of Professor Haddow, is amply recorded in this book. The Editors hope that their intervention has to some extent made for easier reading, but that the reader will be able to enjoy, as if he were a participant, the efforts made by the contributors on this friendly occasion to bring forward new information and to come to an understanding of each other's aims, methods, problems and interpretations.

CONTENTS

List of those participating in or attending the Symposium on "The Influence of Ionizing Radiations on Cell Metabolism" held at the Ciba Foundation, 6th-9th March, 1956

P. Alexander	Chester Beatty Research Inst., London
Tikvah Alper	Experimental Radiopathology Research Unit, Hammersmith Hospital, London
D. W. van Bekkum	Medical Biological Laboratory of the National Defence Research Council T.N.O., Rijswijk
J. Brachet	Laboratoire de Morphologie Animale, Université Libre de Bruxelles
J. A. V. Butler	Chester Beatty Research Inst., London
D. W. Cohn	Oak Ridge National Laboratory, Tennessee, and Chemical Laboratory, University of Cambridge
W. M. Dale	Dept. of Biochemistry, Christie Hospital, and Holt Radium Institute, Manchester
J. N. Davidson	Dept. of Biochemistry, University of Glasgow
A. Forssberg	Inst. of Radiophysics, Stockholm
E. F. Gale	School of Biochemistry, University of Cambridge
L. H. Gray	British Empire Cancer Campaign Research Unit in Radiobiology, Mount Vernon Hospital, Northwood
A. Haddow	Chester Beatty Research Inst., London
G. de Hevesy	Inst. of Organic Chemistry and Biochemistry, University of Stockholm
A. Hollaender	Biology Division, Oak Ridge National Laboratory, Tennessee
Barbara E. Holmes	Dept. of Radiotherapeutics, University of Cambridge
Alma Howard	British Empire Cancer Campaign Research Unit in Radiobiology, Mount Vernon Hospital, Northwood
P. Koller	Chester Beatty Research Inst., London
H. A. Krebs	Dept. of Biochemistry, University of Oxford
L. G. Lajtha	Dept. of Radiotherapy, The Churchill Hospital, Oxford
H. Laser	Molteno Inst., University of Cambridge
R. Latarjet	Laboratoire Pasteur de l'Institut du Radium, Paris

J. F. LOUTIT	Medical Research Council Radiobiological Research Unit, Harwell
J. S. MITCHELL	Dept. of Radiotherapeutics, University of Cambridge
ANTOINETTE PIRIE	Nuffield Laboratory of Ophthalmology, University of Oxford
G. POPJAK	Experimental Radiopathology Research Unit, Hammersmith Hospital, London
S. SPIEGELMAN	Dept. of Bacteriology, University of Illinois
G. E. STAPLETON	Biology Division, Oak Ridge National Laboratory, Tennessee
L. A. STOCKEN	Dept. of Biochemistry, University of Oxford
C. P. SWANSON	Dept. of Biology, The Johns Hopkins University, Baltimore
T. S. WORK	National Inst. for Medical Research, Mill Hill, London
P. C. ZAMECNIK	Massachusetts General Hospital, Boston

CHAIRMAN'S OPENING REMARKS

A. Haddow

A great deal of work has been carried out on the elucidation of the changes in gross cellular structure produced by ionizing radiations, on the histopathology of radiation damage, and on the cytological and genetical effects. Yet what of the biochemical changes, the metabolic changes we have to consider? To quote Dubois and Petersen's review (1954, *Annu. Rev. Nuclear Sci.*, **4**, 351), although research on the biochemical effects of ionizing radiations has yielded a vast amount of information, no satisfactory explanation of the exact mechanism by which tissue damage is inflicted has yet been obtained. Research on the biochemical mechanisms has been under way for a relatively short period of time. A considerable amount of research on the subject during recent years was of necessity exploratory in nature. Many approaches to the problem of mechanism have been employed. A large number of the earlier studies dealt with *in vitro* systems. The information obtained from such studies has been valuable in indicating the chemical linkages and groups which are the most susceptible to alteration by ionizing radiations. However, attempts to apply *in vitro* findings with ionizing radiations to intact cells have been generally disappointing. Biologists have therefore turned their attention to the more difficult task of attempting to define radiation damage in terms of interference with biochemical systems, through research on irradiated animals and microorganisms. My colleague J. A. V. Butler has pointed out that the basic puzzle of radiobiology, one which has been stressed especially by L. H. Gray, is still unsolved—namely that comparatively small doses of radiation produce marked biological changes, although in general rather large doses are required to produce easily observable chemical changes. In Butler's

words, the passage of radiation through living tissues obviously initiates a long chain of events. We have the primary ionizations, the chemical consequences, and the biological events which follow. Although the physical nature of the primary actions has been well worked out, and the chemical consequences have been established, at least in numerous simple cases, the link between the chemical changes and the biological consequences is almost completely unknown. Discussing the radical initiated polymerizations of unsaturated substances, Butler points out that the radical merely acts as a catalytic agent in that it stimulates processes which can occur spontaneously. This recalls a recent impression that the chemical carcinogens may simply expedite processes which occur spontaneously at much lower rates. Again to quote Butler, we are at the moment in the position of a man who tries to elucidate the mechanism of a telephone exchange by throwing bricks into it and observing some of the results.

Our subject is at an elementary stage, yet it is always dangerous to say what will not happen in science. Even Lord Rutherford at one time thought little of the prospects of the release of atomic energy. From the study of the influence of ionizing radiations on cell metabolism may, however, flow the most profound consequences for the theory of ageing, for the theory of carcinogenesis, and for the theory of heredity. J. J. Thomson once said that if he were to start life again he would take up the study of biology, this being, as he thought, at the same stage as physics when he started his early career. Our own subject is the ideal region in which physics, chemistry and biology meet.

CYTOPLASMIC AND NUCLEAR STRUCTURE IN RELATION TO METABOLIC ACTIVITIES

J. Brachet

University of Brussels

Among the numerous theories which have been proposed with a view to explaining the functions of the nucleus in the life of the cell, several have now been definitely rejected. This is true in particular for the hypothesis of Loeb (1899) who considered the nucleus as the prime centre of cellular oxidations, for we have now shown, both for amoebae and for the unicellular alga *Acetabularia mediterranea*, that removal of the nucleus does not appreciably reduce the rate of cellular oxidations, even after a considerable length of time (Brachet, 1955*a*). It is also now well known that isolated nuclei have an extremely low oxygen consumption and lack most of the oxidative enzymes, this being true for amphibian egg nuclei obtained by microdissection (Brachet, 1939) and for nuclei of liver homogenates prepared by differential centrifugation (cf. recent review articles by Dounce, 1955; Allfrey, Daly and Mirsky, 1955). Extensive research on liver homogenates has shown, in addition, that mitochondria are the primary, though not exclusive, site of the energy-generating reactions of the cell (oxidative phosphorylations). This work has been ably summarized in recent reviews by de Duve and his co-workers (de Duve and Berthet, 1954) and by Hogeboom and Schneider (1955). An interesting exception, as yet unconfirmed, has been reported by Rubinstein and Denstedt (1954): bird erythrocytes lack mitochondria and contain oxidative enzymes (cytochrome oxidase and succinic dehydrogenase) in their nuclei.

The fact remains, however, that the metabolism of enucleated cytoplasm is never entirely normal. In the case of

amoebae, removal of the nucleus leads to considerable disturbances of phosphorylation. ^{32}P-incorporation into non-nucleated halves slows down almost immediately (Mazia and Hirshfield, 1950), while their ATP content undergoes an increase in aerobiosis which probably reflects a block in the utilization of the phosphate-bond energy of ATP (Brachet, 1955*a*). Under anaerobic conditions, on the other hand, non-nucleated cytoplasm shows a markedly reduced ability for keeping ATP in phosphorylated form (Brachet, 1955*a*). Moreover, the general metabolic disturbance of non-nucleated cytoplasm is also revealed in other biochemical systems. As we have shown (1955*a*), the utilization of lipid and carbohydrate reserve products is considerably reduced in non-nucleated halves of amoebae.

These metabolic injuries can be accounted for, as we have already suggested (Brachet, 1955*a*), by assuming that the cell nucleus is involved in the synthesis of nucleotide coenzymes, which are essential for glycolysis and cellular oxidations. This hypothesis is in agreement with most recent findings. Hogeboom and Schneider (1952) have shown that, in the liver, the complete enzyme system for the synthesis of diphosphopyridine nucleotide (DPN) from ATP and nicotinamide nucleotide is located in the nuclei. In the starfish oöcyte, as shown by our co-worker E. Baltus (1954), the same enzyme system is concentrated in the nucleoli, which are fifty times more active than entire oöcytes in this respect. If one of the biochemical functions of the cell nucleus consists in the production of DPN-like nucleotide coenzymes, enucleation should result in a rapid loss of these coenzymes from the cytoplasm and Baltus (1956) has found that this is indeed the case: the DPN content of fasted amoebae drops much faster in the non-nucleated than in the nucleated halves.

Certain conclusions can be drawn from these various results. At first it appears that the presence of the nucleus is by no means essential to keep up the normal rate of cellular oxidations and that those cytoplasmic granules which are specially active in cellular oxidations, in particular mitochondria, are

largely independent of the nucleus. The latter, however, does exert an indirect control by regulating these oxidation processes through the synthesis of the nucleotide coenzymes. It appears probable that these coenzymes are protected from hydrolytic enzymes when bound to the mitochondria, in which case removal of the nucleus can have little effect on bound DPN and cannot interfere strongly with cellular oxidations. On the contrary, free coenzymes, those not bound to mitochondria, would appear to be left unprotected against hydrolysis and this should result in a rapid drop of glycolysis with an incomplete utilization of the stored glycogen after removal of the nucleus. Thus non-nucleated cytoplasm, with its low content of free DPN and the resulting deficient glycolysis, should no longer keep up its normal ATP content in anaerobic conditions.

Such a direct action of the nucleus might be postulated not only for the synthesis of DPN, but also for that of the other nucleotide coenzymes (triphosphopyridine nucleotide, flavine-adenine dinucleotide, coenzyme A, etc.). The experiments to prove it have yet to be done but it remains an attractive hypothesis, in view of the extremely important part taken by the nucleus in the metabolism of a polynucleotide, ribonucleic acid (RNA). We already know from ^{32}P experiments by Marshak (1948), Marshak and Calvet (1949), Jeener and Szafarz (1950) and Barnum and Huseby (1950), that nuclear RNA shows a much higher specific activity than cytoplasmic RNA. Studies with other radioactive precursors such as orotic acid (Hurlbert and Potter, 1952), glycine (Bergstrand *et al.*, 1948), formate (Payne *et al.*, 1952; Smellie *et al.*, 1953) have confirmed these results. In all cases, incorporation by nuclear RNA was very high, higher in fact than that by any cytoplasmic fraction.

There has been much debate as to whether, as suggested by Jeener and Szafarz (1950), nuclear RNA is a precursor of cytoplasmic RNA. Recent mathematical work by Barnum, Huseby and Vermund (1953), as well as measurements showing that nuclear and cytoplasmic RNA's have different

molecular compositions (Crosbie, Smellie and Davidson, 1953; Elson, Trent and Chargaff, 1955) give little probability to the idea of nuclear RNA being the sole precursor of cytoplasmic RNA. On the other hand, Goldstein and Plaut (1955) recently succeeded in grafting, in normal and in non-nucleated amoebae, nuclei which had been labelled with ^{32}P. These experiments strongly suggest that nuclear RNA can give rise to cytoplasmic RNA, but they do not demonstrate that nuclear RNA is the sole precursor of cytoplasmic RNA, nor do they prove that nuclear RNA is not degraded prior to its conversion into cytoplasmic RNA. It appears rather as if both forms of RNA are synthesized independently, though at a faster rate in the nucleus than in the cytoplasm. We shall see later that major differences are also found in the fate of RNA in various enucleated organisms.

Let us next consider another aspect of the rôle of the nucleus in the life of the cell, the possible relations of the nucleus with protein synthesis. As early as 1881, Verworn had suggested a control by the nucleus of the cell's anabolism, making this hypothesis in order to explain the usual incapacity of non-nucleated cytoplasm to regenerate. Caspersson (1941, 1950) has taken up this old hypothesis of Verworn and extended it. On cytochemical grounds he has postulated that the nucleus plays a fundamental rôle in protein synthesis, a suggestion we shall now consider in the light of recent experimental results from a number of laboratories.

The observation that cells in which an active protein synthesis goes on have a particularly large nucleolus with a correspondingly high content of RNA, has led Caspersson (1941) to propose that the nucleus, and especially the nucleolus, is a key factor in protein synthesis. Simultaneously with Caspersson (1941) but working independently, we proposed the hypothesis that RNA plays a direct rôle in protein synthesis (Brachet, 1941). This was suggested by the exceptionally high RNA content of all cells actively synthesizing proteins. The hypothesis found further support in the results of Hultin (1950) and of Borsook and co-workers (1952), who

found that microsomes (the smallest cytoplasmic particulates, which have also the highest RNA content) are most active in the incorporation of radioactive amino acid into proteins. More recently, Gale and Folkes (1954, 1955) have found, in bacteria lysed by ultrasonics, that protein synthesis will only take place if RNA is left intact. Indeed this process is brought to a stop if the nucleic acid fraction is extracted by various means. In our laboratory also (Brachet, 1954, 1955*a* and *b*), it has been shown that ribonuclease, by specifically attacking or binding the RNA of normal, living cells (onion roots, amoebae, star-fish or amphibian eggs, etc.), has a powerful inhibitory action on the incorporation of amino acids into proteins, on the growth of the cell and on its overall protein synthesis.

It is now a generally accepted fact, as pointed out by Borsook (1955), Gale (1955) and Mirsky (1955), that nucleic acids are directly and fundamentally involved in protein synthesis. This is clear at least in the case of RNA (Gale and Folkes, 1954, 1955; Brachet, 1954, 1955 *a* and *b*), but appears less evident for DNA; some experiments of Allfrey (1954) and Allfrey and Mirsky (1955) do indicate that desoxyribonuclease inhibits amino acid incorporation into the proteins of isolated thymus nuclei; the inhibition, however, is not so strong as that by ribonuclease for the whole cell although ribonuclease does not inhibit amino acid incorporation into the proteins of isolated nuclei.

In view of the high nucleic acid content of cell nuclei and because of the now well-established importance of these compounds in protein synthesis, Caspersson's idea (1941, 1950) of a particularly important function of the nucleus in protein synthesis has been brought into focus again and several laboratories have initiated experiments on this problem. A simple method, used chiefly by Mirsky and co-workers (Daly, Allfrey and Mirsky, 1952) and by Davidson and co-workers (Crosbie, Smellie and Davidson, 1953; Smellie, McIndoe and Davidson, 1953), consists in injecting a radioactive amino acid into a living animal and then determining the specific

radioactivity of the various constituents of its liver cells (nuclei, mitochondria, microsomes and supernatant as obtained by differential centrifugation). This technique is open to some criticism. Results of the Mirsky group show that the methods of preparation of isolated nuclei used entail serious losses of some nuclear proteins. In this manner histones have been shown to incorporate amino acids only slowly, while the rest of the nuclear proteins do not differ much in activity from the whole of cytoplasmic proteins. But it must always be kept in mind that the current preparation processes may well extract from the nuclei some proteins of considerable metabolic importance.

In the light of these objections, we have taken up a different aspect of the same problem, one which appears more worthwhile from a biologist's standpoint. Together with a group of co-workers, we have investigated protein metabolism in nucleated and non-nucleated halves of unicellular organisms. We have deliberately selected two widely separated species: the amoeba (*Amoeba proteus*), the non-nucleated halves of which cannot regenerate, and the giant unicellular alga, *Acetabularia mediterranea*, in which the non-nucleated stems remain capable of extensive regeneration, as shown by the classical work of Hämmerling (1934, 1953). We shall next consider the results obtained in both cases.

If one cuts an amoeba into half, the non-nucleated fragment soon rounds up and stops feeding. The nucleated half keeps behaving normally and, if fed living micro-organisms, it can resume growth and divide. Since the biochemical changes in both halves should be studied under comparable conditions, both fragments must be kept fasting in the course of the experiment. Under these conditions the non-nucleated halves remain alive for 10–15 days and the nucleated fragments for about 3 weeks.

Our experiments have led us to the following conclusions. As already pointed out, the oxygen consumption of non-nucleated halves remains unaffected, but their ATP content rises aerobically. Under anaerobic conditions, however,

anucleate cytoplasm cannot keep up its normal ATP content for long. We already know that these non-nucleated fragments rapidly lose the ability to utilize stored glycogen (Brachet, 1955*a*). Similarly they stop incorporating ^{32}P (Mazia and Hirshfield, 1950) and cannot maintain a normal DPN content (Baltus, 1956). This leaves no doubt that the loss of the nucleus leads to serious defects of the metabolism of carbohydrates and phosphorylated compounds. A logical consequence of such an inhibition of the energy-providing mechanisms of the cell would be a severe disturbance of protein synthesis, since it is well established that the synthesis of proteins or even of simple peptide bonds requires energy from the high energy phosphate bonds of ATP (Borsook, 1950; Siekevitz, 1952; etc.).

With respect to protein synthesis, removal of the nucleus brings into play an additional unfavourable factor in the amoeba: we have already mentioned that RNA is also involved in this synthesis. Removal of the nucleus, as shown in 1951 by Linet and Brachet and, in confirmation, by James (1954), leads to an immediate and marked fall in the RNA content of cytoplasm (this can reach 70 per cent within ten days). Such a drop in cytoplasmic RNA is, of course, in agreement with the more recent autoradiograph experiments of Goldstein and Plaut (1955), showing that at least some of the cytoplasmic RNA in amoebae is of nuclear origin. It is not surprising that total protein content decreases faster in non-nucleated than in nucleated halves (Linet and Brachet, 1951).

Given the conditions of our experiments (complete fasting), one could not, of course, expect net protein synthesis to occur. A process very near to it could be followed, however: the incorporation of tagged amino acids into proteins. In a recent paper, Mazia and Prescott (1955) have shown that removal of the nucleus leads, within as little as 2 or 3 hours, to a drastic decrease in the uptake of radioactive methionine by the proteins of non-nucleated amoebae. The N/A ratio (N = nucleated half; A = anucleate half) is already increased

to a value of 6 after a few hours, and it reaches the value of 20 at the end of 2–3 days. We must bear in mind, however, that methionine uptake by the proteins of non-nucleated halves never drops to zero. Such uptake is strongly diminished, but never abolished by removal of the nucleus.

More recently, in experiments done with our co-worker Mrs. A. Ficq, in which radioactive phenylalanine was used as a precursor and located in the cell by autoradiography, we have essentially confirmed Mazia and Prescott's results. The differences we observed are less striking, however, since our N/A ratios are in the neighbourhood of 2 (instead of 6–20) from 1 to 6 days after removal of the nucleus in the amoeba and in the neighbourhood of 5 after 10 days. This is taken to mean that, in the amoeba, removal of the nucleus does not immediately stop protein metabolism in the cytoplasm. As a matter of fact, the amino acid uptake by proteins of the non-nucleated halves begins markedly to decrease only when the RNA content of the non-nucleated cytoplasm is already much diminished (Linet and Brachet, 1951).

Thus we come to the notion that, in the case of the amoeba at least, the nucleus cannot be the exclusive centre of protein synthesis. Amino acid incorporation into proteins is maintained at a non-negligible rate in non-nucleated fragments as long as the RNA content of the latter remains essentially unchanged. The same experiments, on the other hand, show very clearly that the nucleus exerts a control on protein metabolism in the cytoplasm. This gives rise to another problem: are all the cytoplasmic proteins equally dependent on the nucleus?

This was investigated, still using amoebae, by following in the course of time the changes of various enzymes (hence of as many distinct proteins) in both types of fragments. The results were essentially as follows (Brachet, 1955*a*). In the amoeba, the removal of the nucleus results in widely different effects in the case of different enzymes. Some enzymes, such as protease, amylase and enolase, remain practically unchanged after removal of the nucleus; others, dipeptidase for

instance, show a very slow decrease in the non-nucleated halves; a third group, including esterase and acid phosphatase, have practically disappeared from non-nucleated cytoplasm after a few days. This establishes without any doubt that different enzymes are to different extents under nuclear control and that this postulated "control" from the nucleus is much more complex than was expected at first.

It is still too early to state definitely why the various enzymes we studied behave so differently after removal of the nucleus; a likely explanation, though lacking formal proof as yet, might be the different cytological localizations of these enzymes; as shown by Holter and Løvtrup (1949), in the amoeba, amylase and protease are bound to large mitochondrion-like particles. This would imply that mitochondria are, by and large, independent of the nucleus. This is, moreover, in perfect agreement with the finding, reported above, that removal of the nucleus has little effect on the rate of cellular oxidations. According to Holter and Pollock (1952), dipeptidase is found in solution in the hyaloplasm; it is therefore not surprising that it should behave, after removal of the nucleus, like the whole of the proteins of the organism. Finally, acid phosphatase and esterase both show a striking decrease in activity in non-nucleated cytoplasm, just like RNA; it is not unlikely, therefore, that we are here dealing with microsome-bound enzymes. If this proves to be the case, it would mean that these small cytoplasmic granules are under much closer nuclear control than the mitochondria and the soluble proteins of the hyaloplasm.

In summary, removal of the nucleus in the amoeba is followed essentially by a drastic decrease of DPN, RNA, acid phosphatase and esterase, a marked fall in the incorporation of amino acids into proteins, a loss of the ability to retain phosphorylated ATP under anaerobic conditions, and a slow decrease of the total protein and dipeptidase content. On the other hand, removal of the nucleus hardly changes oxygen consumption, aerobic ATP, protease, amylase and enolase.

Parallel studies done on the unicellular alga *Acetabularia mediterranea*, an organism which can regenerate to a sizeable extent when deprived of its nucleus (Hämmerling, 1934), have yielded quite different results. As we have shown recently (Brachet, Chantrenne and Vanderhaeghe, 1955), *Acetabularia mediterranea* behaves much like the amoeba as far as respiration is concerned. In both, removal of the nucleus has no measurable effect on cellular oxidations, showing that the latter are not under direct nuclear control. Indeed, the reverse appears to be true to some extent in *Acetabularia*. The morphology and chemical composition of the nucleus are influenced by the cytoplasm. If energy production in the cytoplasm is diminished or blocked by dinitrophenol or anaerobiosis, the nucleolus soon changes its shape, losing in the process some of its high content of RNA. Such a nucleolar reaction has been observed before by Stich (1951) as a result of merely placing the algae in the dark.

We have, however, noted a difference between *Amoeba* and *Acetabularia* for phosphorus metabolism. While it is true that ^{32}P incorporation decreases in non-nucleated *Acetabularia* stems, this effect does not become noticeable until after a long time (Brachet, Chantrenne and Vanderhaeghe, 1955), usually several weeks. Fragments separated for only a few days show no significant differences in this respect (Hämmerling and Stich, 1954).

It is, however, with respect to RNA and protein metabolism that *Acetabularia* shows most difference from what has been reported for *Amoeba*. We have recently been able to show (Brachet, Chantrenne and Vanderhaeghe, 1955) that the non-nucleated stem of *Acetabularia* retains for several weeks the ability to incorporate radioactive $^{14}CO_2$ into proteins (in the light) and orotic acid into RNA. These anabolic processes continue at a normal rate for fifteen days in non-nucleated cytoplasm. Together with regenerative potency, they then begin to diminish. Even after three months without a nucleus, fragments will still be capable of a noticeable uptake of radioactive precursors into RNA and proteins.

Even more striking is the fact that non-nucleated cytoplasm can actually effect a net synthesis of proteins and RNA. Indeed, during the first days after halving, this simultaneous synthesis of RNA and proteins is even more rapid in the non-nucleated than in the nucleated half. Perhaps this is due to the fact that the nucleus competes with the cytoplasm for ribonucleoprotein precursors. If the nucleus utilizes these precursors at a higher rate than does the cytoplasm (we shall later see that it could very well be so), the acceleration of net RNA and protein synthesis with removal of the nucleus is easy to understand.

The fact that net protein and RNA synthesis is possible in the absence of the nucleus has interesting implications: for instance, it is clear that, in contradiction to one of the theories we have reviewed above, cytoplasmic RNA does not necessarily originate in the nucleus. Furthermore, if RNA is organized under the influence of DNA as has been suggested by Gale and Folkes (1954), it is obvious that such a mechanism must be a remote one. In *Acetabularia*, RNA synthesis is certainly possible in the absence of DNA and the experiments show that simple ideas such as "DNA makes RNA, and RNA makes protein" are the result of an oversimplification of the facts.

It remains, however, that this RNA and protein synthesis in the absence of the nucleus does not go on indefinitely: the process gradually slows down after 10 days or so. This shows again that the nucleus does exert some control on protein synthesis in the cytoplasm, but this control is remote and indirect, not immediate as might have been expected.

The chemical nature of the nuclear control exerted by the nucleus on protein synthesis is still unknown. It might be that, as in Goldstein and Plaut's (1955) experiments with amoebae, part of the cytoplasmic RNA originates from the nucleus in *Acetabularia* also and protein synthesis might come to a standstill when this RNA of nuclear origin has been exhausted. It would be important to know whether the RNA, which is so quickly synthesized by the non-nucleated

Acetabularia stems, has the chemical composition of the nuclear RNA or that of the cytoplasmic RNA, provided the two differ in this respect; for the essential fact remains that the non-nucleated *Acetabularia mediterranea* is capable of forming a specific *mediterranea* regenerate in the absence of a nuclear production of either RNA or DNA. It is unlikely that the morphogenetic substance produced by the *Acetabularia* nucleus is DNA, since we have been unable to detect the presence of DNA in the non-nucleated stems with a sensitive isotope dilution method.

The observed differences, with respect to protein and RNA synthesis in non-nucleated cytoplasm, between *Amoeba* and *Acetabularia* are not altogether unexpected if we consider that the non-nucleated stem of an *Acetabularia* retains, as we have seen for ourselves, a perfectly normal photosynthetic activity. Thus the energy requirements for nucleoprotein synthesis are still met with in a non-nucleated piece of *Acetabularia*, but not in a non-nucleated *Amoeba* half.

We must finally point out the very clear correlation which is found in both *Amoeba* and *Acetabularia* between the fate of RNA and that of the proteins. In the former, removal of the nucleus is followed by a rapid loss of RNA and a marked decrease in protein metabolism. In *Acetabularia*, on the contrary, both processes are accelerated in a parallel manner. Those are by no means special cases, since what we have just said for *Acetabularia* applies also to reticulocytes. These are immature red cells which have lost their nucleus, but still retain a nearly normal amount of RNA in the cytoplasm. They still have the power of incorporating tagged amino acids into their proteins, an activity which is completely lacking in mature erythrocytes. The latter have almost completely lost their RNA (Borsook *et al.*, 1952; Koritz and Chantrenne, 1954; Holloway and Ripley, 1952). The same reticulocytes, in spite of the loss of their nucleus, can also incorporate radioactive glycine in their RNA, as shown recently by Kruh and Borsook (1955); these anucleate cells can even synthesize haemoglobin (Nizet and Lambert, 1953) and various enzymes

(Koritz and Chantrenne, 1954). This ability of reticulocytes to synthesize proteins and to incorporate radioactive precursors into their proteins and RNA, decreases as they lose their basophilia, i.e. their RNA, in the course of the maturation process. This correlation between the drop in basophilia and the decrease in glycine uptake into proteins is particularly obvious in autoradiographic observations by Gavosto and Rechenmann (1954): their technique allowed both processes to be followed simultaneously.

These results on reticulocytes are in full agreement with the data from *Acetabularia*: removal or spontaneous elimination of the nucleus does not necessarily lead to a rapid block of protein synthesis. As a matter of fact, the results of the experiment are chiefly dependent on the effects on cytoplasmic RNA brought about by removal of the nucleus. If cytoplasmic RNA is quickly broken down in the absence of the nucleus, as happens in *Amoeba*, protein metabolism is immediately affected. If, on the other hand, the non-nucleated cytoplasm keeps its normal RNA content for a long time (as in *Acetabularia* and reticulocytes), it can still synthesize proteins, at least for a while.

Similar instances could no doubt be found in the case of eggs deprived of their nucleus: unfortunately the data gathered in this field so far [by Malkin (1954) on sea urchin eggs and by Tiedemann and Tiedemann (1954) on Triton eggs], lack a sufficiently complete analysis. We can at least state, for both the sea urchin and the newt, that the non-nucleated half is no less potent than the nucleated fragment with respect to incorporating radioactive precursors into proteins and RNA. It is a fact, however, that in unfertilized eggs, as used in the above experiments, the net synthesis of proteins and RNA is likely to be negligible. It follows that those results of Malkin (1954) and Tiedemann and Tiedemann (1954) should probably be taken as meaning that the turnover of RNA and proteins remains at its normal level in non-nucleated egg cytoplasm.

From the data available so far, we can now draw a general

conclusion: the nucleus exerts, at the most, only a remote and delayed control on the synthesis of cytoplasmic proteins. It does not necessarily follow that the actual rôle of the nucleus is negligible in the synthesis of proteins in the whole, intact cells. We have already recalled the results from the laboratories of Mirsky (Daly, Allfrey and Mirsky, 1952) and of Davidson (Crosbie, Smellie and Davidson, 1953; Smellie, McIndoe and Davidson, 1953) in which it was established that some nuclear proteins take up marked amino acids at a rate comparable to that of the whole of cytoplasmic proteins. We have pointed out on this occasion that the methods used in isolating these nuclei may well involve the loss of soluble proteins which might be very active metabolically.

This criticism is motivated by some recent autoradiographic observations. When amino acid incorporation into proteins is followed by this method, a much higher activity is found in the nucleus than in the cytoplasm. This has been shown for various materials: growing oöcytes (Ficq, 1953), amphibian eggs in the course of their development (Ficq, 1954; Sirlin, 1955) and mammalian liver (Ficq and Errera, 1955; Moyson, 1955). This higher activity of the nuclei becomes much less obvious when the liver sections are extracted by dilute citric acid, as used for the isolation of nuclei. It appears possible, therefore, that this acid extracts some metabolically active proteins from the nuclei. This might be shown conclusively by a study of radioactive amino acid incorporation into nuclei isolated in non-aqueous media.

These autoradiographic studies have not been carried out to a sufficient extent to allow general conclusions to be drawn from them. It is by no means certain that the nuclei of all cells are more active in this manner than the cytoplasm: that has only been shown, so far, for cells with a high rate of protein synthesis (oöcytes, livers) and for actively dividing cells (embryos in the course of development).

The autoradiographic experiments of Ficq (1953, 1955) on starfish oöcytes are more informative. In this material, it

is the nucleolus which has the most rapid and considerable uptake of the tagged precursors (adenine, phenylalanine) of RNA and proteins. This is most marked in young, actively growing oöcytes in which considerable nucleoprotein synthesis is going on. In such oöcytes, the activity of the nucleolus can be a hundred times that of the other constituents of the cell (nuclear sap, cytoplasm). Similar results for RNA have also been published by Taylor (1953) and by Stich and Hämmerling (1953), using ^{32}P as a precursor: the former using autoradiographic methods, the latter measuring the activity in the giant nucleolus of *Acetabularia*, isolated by microdissection.

We can therefore conclude that the nucleus, especially the nucleolus, is the site of a particularly active protein and RNA metabolism; this metabolism, however, can remain unaffected for a long time in non-nucleated cytoplasm; net synthesis of proteins and RNA can even take place in such cytoplasm. In all known cases, a very strict parallel has been found between the fate of RNA and that of protein anabolism. Such a parallel lends support to the hypothesis put forth by Caspersson (1941) and ourselves (1941) that RNA is directly involved in protein synthesis.

Summary

It has now been conclusively shown that the nucleus is not a prime centre of cellular oxidations. It seems, nevertheless, that the nucleus plays a fundamental rôle in the synthesis of nucleotides and of ribonucleic acid (RNA) and it may be that the nucleus, and especially the nucleolus, is directly concerned in the synthesis of nucleotide coenzymes.

It is certain that the nucleus plays an important rôle in protein synthesis, although in the unicellular alga *Acetabularia* protein synthesis can go on for long periods without a nucleus. Indeed, non-nucleated fragments of *Acetabularia* are able to synthesize RNA and proteins for some fifteen days. The rate of these syntheses decreases afterwards, showing that the

nucleus exerts a very real, but remote, control on the production of cytoplasmic proteins. These experiments also demonstrate that cytoplasmic RNA cannot originate exclusively from nuclear RNA.

Cases of closer control by the nucleus of protein synthesis are shown by reticulocytes and especially by amoebae, in which removal of the nucleus leads to a rapid decrease in the incorporation of tagged amino acids into proteins and a drop in RNA content of the cytoplasm. In the amoebae, removal of the nucleus has different effects on various enzymes. It appears that those enzymes bound to microsomes are particularly affected by the removal of the nucleus, while mitochondrial enzymes are especially independent of the presence or absence of the nucleus.

Finally, autoradiographic experiments have shown that, in cells in which a high mitotic activity or an active protein synthesis goes on, the nucleus is more active than the cytoplasm in incorporating tagged amino acids into its proteins.

Acknowledgement

The author wishes to thank Dr. P. Couillard, who kindly translated from the French text.

REFERENCES

ALLFREY, V. G. (1954). *Proc. nat. Acad. Sci., Wash.*, **40**, 881.
ALLFREY, V. G., DALY, M., and MIRSKY, A. E. (1955). *J. gen. Physiol.*, **38**, 415.
ALLFREY, V. G., and MIRSKY, A. E. (1955). *Nature, Lond.*, **176**, 1042.
BALTUS, E. (1954). *Biochim. biophys. acta*, **15**, 263.
BALTUS, E. (1956). *Arch. int. Physiol.*, **64**, 124.
BARNUM, C. P., and HUSEBY, R. A. (1950). *Arch. Biochem.*, **29**, 7.
BARNUM, C. P., HUSEBY, R. A., and VERMUND, H. (1953). *Cancer Res.*, **13**, 880.
BERGSTRAND, A., ELIASSON, N. A., NORBERG, B., REICHARD, P., and UBISCH, H. VON (1948). *Cold Spr. Harb. Symp. quant. Biol.*, **13**, 22.
BORSOOK, H. (1950). *Physiol. Rev.*, **29**, 206.
BORSOOK, H. (1956). Proc. III Int. Congr. Biochem., p. 92. New York: Academic Press.
BORSOOK, H., DEASY, C. L., HAAGEN-SMIT, A. J., KEIGHLEY, O., and LOWY, P. H. (1952). *J. biol. Chem.*, **196**, 669.
BRACHET, J. (1939). *Arch. exp. Zellforsch.*, **22**, 541.

BRACHET, J. (1941). *Arch. Biol., Paris*, **53**, 207.
BRACHET, J. (1954). *Nature, Lond.*, **174**, 876.
BRACHET, J. (1955*a*). *Biochim. biophys. acta*, **18**, 247.
BRACHET, J. (1955*b*). *Nature, Lond.*, **175**, 851.
BRACHET, J., CHANTRENNE, H., and VANDERHAEGHE, F. (1955). *Biochim. biophys. acta*, **18**, 544.
CASPERSSON, T. (1941). *Naturwissenschaften*, **29**, 33.
CASPERSSON, T. (1950). Cell Growth and Cell Function. New York: Norton.
CROSBIE, G. W., SMELLIE, R. M. S., and DAVIDSON, J. N. (1953). *Biochem. J.*, **54**, 287.
DALY, M., ALLFREY, V. G., and MIRSKY, A. E. (1952). *J. gen. Physiol.*, **36**, 173.
DE DUVE, C., and BERTHET, J. (1954). *Int. Rev. Cytology*, **3**, 225.
DOUNCE, A. L. (1955). *In* The Nucleic Acids, p. 93. New York: Academic Press.
ELSON, D., TRENT, L. W., and CHARGAFF, E. (1955). *Biochim. biophys. acta*, **17**, 362.
FICQ, A. (1953). *Experientia*, **9**, 377.
FICQ, A. (1954). *J. Embryol. exp. Morphol.*, **2**, 194.
FICQ, A. (1955). *Arch. Biol., Paris*, **66**, 509.
FICQ, A., and ERRERA, M. (1955). *Biochim. biophys. acta*, **16**, 45.
GALE, E. F. (1956). Proc. III Int. Congr. Biochem., p. 345. New York: Academic Press.
GALE, E. F., and FOLKES, J. P. (1954). *Nature, Lond.*, **173**, 1223.
GALE, E. F., and FOLKES, J. P. (1955). *Biochem. J.*, **59**, 675.
GAVOSTO, F., and RECHENMANN, R. (1954). *Biochim. biophys. acta*, **13**, 583.
GOLDSTEIN, L., and PLAUT, W. (1955). *Proc. nat. Acad. Sci., Wash.*, **41**, 874.
HÄMMERLING, J. (1934). *Arch. EntwMech. Org.*, **131**, 1.
HÄMMERLING, J. (1953). *Int. Rev. Cytology*, **2**, 475.
HÄMMERLING, J., and STICH, H. (1954). *Z. Naturf.*, **9b**, 1949.
HOGEBOOM, G. H., and SCHNEIDER, W. C. (1952). *J. biol. Chem.*, **197**, 611.
HOGEBOOM, G. H., and SCHNEIDER, W. C. (1955). *In* The Nucleic Acids, p. 199. New York: Academic Press.
HOLLOWAY, B. W., and RIPLEY, S. H. (1952). *J. biol. Chem.*, **196**, 695.
HOLTER, H., and LØVTRUP, S. (1949). *C.R. Lab. Carlsberg*, **27**, 27.
HOLTER, H., and POLLOCK, B. M. (1952). *C.R. Lab. Carlsberg*, **28**, 211.
HULTIN, T. (1950). *Exp. Cell Res.*, **1**, 376.
HURLBERT, R. B., and POTTER, V. R. (1952). *J. biol. Chem.*, **195**, 257.
JAMES, T. W. (1954). *Biochim. biophys. acta*, **15**, 367.
JEENER, R., and SZAFARZ, D. (1950). *Arch. Biochem.*, **26**, 54.
KORITZ, S. B., and CHANTRENNE, H. (1954). *Biochim. biophys. acta*, **13**, 209.
KRUH, J., and BORSOOK, H. (1955). *Nature, Lond.*, **175**, 386.
LINET, N., and BRACHET, J. (1951). *Biochim. biophys. acta*, **7**, 607.
LOEB, J. (1899). *Arch. EntwMech. Org.*, **8**, 689.
MALKIN, H. M. (1954). *J. cell. comp. Physiol.*, **44**, 105.

MARSHAK, A. (1948). *J. cell. comp. Physiol.*, **32**, 381.
MARSHAK, A., and CALVET, F. (1949). *J. cell. comp. Physiol.*, **34**, 451.
MAZIA, D., and HIRSHFIELD, H. (1950). *Science*, **112**, 297.
MAZIA, D., and PRESCOTT, D. M. (1955). *Biochim. biophys. acta*, **17**, 23.
MIRSKY, A. E. (1956). Proc. III Int. Congr. Biochem., p. 349, New York: Academic Press.
MOYSON, F. (1955). *Arch. Biol., Paris*, **66**, 149.
NIZET, A., and LAMBERT, S. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 771.
PAYNE, A. H., KELLY, L. S., BEACH, G., and JONES, H. B. (1952). *Cancer Res.*, **12**, 5426.
RUBINSTEIN, D., and DENSTEDT, O. F. (1954). *Canad. J. Biochem. Physiol.*, **32**, 548.
SIEKEVITZ, P. (1952). *J. biol. Chem.*, **195**, 549.
SIRLIN, J. L. (1955). *Experientia*, **11**, 112.
SMELLIE, R. M. S., MCINDOE, W. M., and DAVIDSON, J. N. (1953). *Biochim. biophys. acta*, **11**, 559.
STICH, H. (1951). *Z. Naturf.*, **6b**, 319.
STICH, H., and HÄMMERLING, J. (1953). *Z. Naturf.*, **8b**, 329.
TAYLOR, J. H. (1953). *Science*, **118**, 555.
TIEDEMANN, H., and TIEDEMANN, H. (1954). *Naturwissenschaften*, **41**, 535.
VERWORN, M. (1881). *Pflüg. Arch. ges. Physiol.*, **51**, 1.

DISCUSSION

Gray: Prof. Brachet, do you know the actual time-scale in which DPN disappears after removal of the nucleus?

Brachet: Yes, there is a fall of about 25 per cent in the DPN content of *Amoeba* within 24 hours. It is a fast phenomenon. We have not done it yet in *Acetabularia*.

Gray: Does DPN disappear both under aerobic and anaerobic conditions?

Brachet: Our observations were made under aerobic conditions. It will be difficult to test the effects of anaerobic conditions because, in order to measure the DPN content of *Amoeba*, using a very sensitive method, we have to cut about 4–5,000 amoebae for each experiment. What we would really like to know is whether the *distribution* of DPN would be altered in non-nucleated cytoplasm: there is a possibility that part of the DPN may be linked to mitochondria. Perhaps only the free DPN is attacked, while the DPN bound to the mitochondria is more or less preserved. It may be that this loss of DPN, especially if it is soluble DPN, is one of the causes of the poor utilization of carbohydrate reserves.

Davidson: I should like to raise one point in connection with nuclear RNA: my colleagues and I believe that nuclear RNA is heterogeneous, that at least two types of RNA, not just a single RNA, are present in the nucleus. If we isolate cell nuclei by methods which avoid the use of aqueous media, we can obtain perfectly clean cell nuclei from which

we can extract both protein and RNA by means of dilute citric acid. If we use labelled nuclei isolated from an animal that has received radioactive phosphorus beforehand, then the RNA which is extracted from the isolated nuclei has a lower specific activity than the RNA which remains. We do not yet know whether there is any difference in base ratios, but there certainly is a difference in specific activity, and moreover, both the easily extractable and the non-extractable RNA have specific activities which differ from those of any of the RNA's of the cell cytoplasmic fraction. We have done the same sort of thing with nuclei which have been isolated in sucrose-$CaCl_2$ media. Whether we label the animals with radioactive phosphorus or radioactive carbon in the form of ^{14}C-formate, when the nuclei are treated with dilute citric acid or dilute phosphate buffer, an RNA can be extracted which has a different specific activity from that of the RNA which remains, and a different specific activity from that of any of the cytoplasmic fractions. So here we have some fairly substantial evidence that the RNA of the nucleus is heterogeneous.

One might, of course, argue that the easily extractable RNA is simply cytoplasmic RNA which is adhering to the nuclei. Against this is the fact that nuclei prepared by the methods which we use are very clean indeed according to microscopic examination. We have had them examined microscopically by critics who were doing their best to find flaws in our technique and they failed to do so, so that we do believe that the RNA which is extracted is essentially nuclear RNA.

The possibility of nuclear RNA being the precursor of cytoplasmic RNA is extremely interesting, but as Prof. Brachet said, the evidence for a direct move of RNA from nucleus to cytoplasm is not good at the moment.

Brachet: The localization of these two RNA's in the nuclei is not known. If you study it by means of interference microscopy—I suppose there is enough RNA which can be removed by the treatment with citric acid—you might find out where it is located. There may be one difficulty in this work on analysis of bases, i.e. it is difficult to get homogeneous RNA fractions, even from the tissues; so that it may be that in the case of nuclei which have already been isolated, for instance by extraction with citric acid, one may not get the true specific activity. It may be a biochemical artifact to a certain extent.

Butler: What is the evidence for the presence of RNA in chromatin?

Brachet: As far as I know, there are only two pieces of evidence for RNA in chromatin: one is that with cytochemical methods one finds that there are differences in the staining ability of the nuclei from different tissues. As a rule, the nuclei of tissues which do not synthesize proteins stain almost green with methyl green-pyronin. In the case of liver, pancreas, etc., there is a lilac or violet colour of the nuclei. If you treat the sections with ribonuclease, you will find that the nuclei now stain completely green. The red colour of the chromatin disappears as well as the red colour of the cytoplasm and the nucleolus. Furthermore, Mirsky and Ris, in their work on isolated chromosomes, obtained some RNA in the threads.

Davidson: Are you keeping in mind that Dounce (1955, *In* The Nucleic Acids, ed. E. Chargaff and J. N. Davidson, Vol. II, p. 147) found that in isolated rat liver the nucleic acid was mainly DNA, not RNA? So where can the nuclear RNA be in the rat but in the nucleolus, unless it be in the chromatin?

Brachet: I wonder how much of Dounce's material consisted of nucleoli.

Butler: If you isolate the chromatin threads it is quite difficult in group preparations to detect any RNA. I don't happen to remember the figure of Mirsky.

Brachet: It came to about 10 per cent of the DNA content.

Butler: I should say we do not find that in our analyses of thymus DNA.

Brachet: Thymus would be rather different from liver, because thymus nuclei stain almost completely green with methyl green-pyronin.

Butler: DNA from normal rat livers is also effectively free from RNA.

Holmes: Dr. Jacobson (Jacobson, W., and Webb, M. (1952), *Exp. Cell Res.*, **3**, 153) showed by staining methods that, as prophase begins, ribonucleoprotein is added to the outside of the chromosome. This remains during metaphase and anaphase, but in late anaphase ribonucleoprotein appears to be shed from the chromosomes into the cytoplasm between the two groups of chromosomes. By late telophase this ribonucleoprotein has disappeared from the nucleus. The amount found must depend a little on whether the tissue is a dividing or a resting tissue, and on the state of the nuclei.

Brachet: I have had an experience similar to that of Dr. Jacobson. There certainly are changes in the staining ability of the chromosomes during the mitotic cycle, but it is very difficult to know exactly what is happening unless one makes quantitative estimations. The shape of the chromosomes changes so much that it becomes extremely difficult to decide whether or not there is an increase of a substance. This dilution effect is dangerous in cytochemical work.

Popjak: Since DPN synthesis is confined to the nucleus, it is not surprising that the enucleated part of the amoeba eventually runs down, because one of the primary acceptors of electrons from the various substrates is gradually eliminated, and that is why vital functions cannot proceed. In that connection, therefore, I wonder to what extent we can ascribe a function to nuclear DNA or nuclear RNA on the one hand and to the running down of DPN on the other, in the changes of metabolism of cytoplasm.

With regard to the experiments on the transfer of labelled nuclei, where the labelling was with ^{32}P, is there any other evidence that the label that subsequently appears in the cytoplasm is in fact associated with RNA?

Brachet: With regard to the second point, it was done by an autoradiograph method. With such a method, it is likely that many soluble phosphorus-containing substances are lost. Since, after using fixative,

staining, etc., Goldstein and Plaut found that all of the label could be removed both from the nucleolus and the cytoplasm by ribonuclease, it seems that what has been marked really is RNA. But I do not think that the experiment shows more than the fact that cytoplasmic RNA is labelled under these conditions. We cannot be sure that the nuclear RNA has gone directly into the cytoplasm, because it is quite possible that intermediary biochemical stages break down this RNA. Goldstein and Plaut say that RNA does not go the other way round, i.e. from the cytoplasm to the nucleus. But, to be really sure, a normal nucleus should be introduced into very strongly labelled cytoplasm, and this experiment has not been done.

As regards Dr. Popjak's first question, I believe that quite a number of changes occur in a non-nucleated *Amoeba*. Why they ultimately die off, I do not know, but this is a slow, progressive process; the overall lifespan is always somewhat lower without the nucleus than with it and the same applies to *Acetabularia*. The very first changes which occur are unfortunately not known to us: when you cut an *Amoeba* in half, you can identify the enucleated half 15 minutes later, because it has rounded up; there is something going on very quickly in the membrane which is not yet understood.

Howard: Prof. Brachet, what would happen if you added the nucleus of one species of *Acetabularia* to the non-nucleated half of a different species? Would it grow a hat of the species from which the nucleus came?

Brachet: I don't think the experiment has been done. The only thing that has been done by Hämmerling is the transfer of a nucleus between two species and what then happens is this: if you cut *Acetabularia mediterranea* just before the hat is formed, you will get a small, but typical *Acetabularia mediterranea* hat. If you graft a nucleated half of another species—for instance *Acetabularia crenulata*—in the stem of *Acetabularia mediterranea* before the formation of the hat, then you get hybrid hats. The purpose of the hat is the reproduction of the alga. This hybrid hat is never fertile; it can be replaced by a second hat, which will now be a typical *crenulata* hat.

These experiments of Hämmerling show that there are morphogenetic substances produced under the influence of the nucleus; whether they originate from the nucleus or are due to nucleocytoplasmic interactions, we do not know.

Koller: You mentioned that in *Acetabularia* when you cut off the hat, the enucleated stem will develop another hat of the same kind as the original. This shows that protein synthesis in the enucleated part is still under genetic control. It would be interesting to see how far genetic control remains in operation. By removing the regenerated hat from the enucleated *Acetabularia* and repeating the process it might be possible to distinguish morphogenetic substances which are derived from the nucleus from those which are produced in the enucleated part.

Brachet: I cannot answer that question because we have not done the experiment. I believe that, probably very soon after the hat has been formed, you will no longer be able to form a hat. We have carried out experiments where we have tried to find out how long the non-nucleated

half is capable of regenerating a hat. This experiment is very simple: you cut a number of algae, take the non-nucleated halves. Light is needed to provide energy for the regeneration. You put some of the algae immediately in the light, you keep another batch for one week, another for two weeks or three–four weeks in the dark before exposing them to the light. When you put a batch of non-nucleated algae immediately in the light, you may get about 70 per cent hats. If you leave them one or two weeks in the dark, you still get 70 per cent hats; but if you leave them three weeks in the dark, you get only 25 per cent hats, and after four weeks you have none. There is something which dies off in the dark as well as in the light. The time when the percentage of the hats decreases (i.e. two weeks) is the same when net protein synthesis and incorporation of $^{14}CO_2$ in the proteins also decrease in the light.

THE EFFECTS OF IONIZING RADIATIONS ON ENZYMES *IN VITRO*

W. M. Dale

Department of Biochemistry, Christie Hospital and Holt Radium Institute, Manchester

One important task for the biologist is to correlate the radiation effects obtained in experiments *in vitro* with those in living matter. Since enzymes are essential constituents of cells, this short survey of radiation effects on enzymes *in vitro* is meant to form a background against which the action of radiation *in vivo* should be viewed, and I hope that the following papers and their discussion will open new ways of approach to decide what part enzymes may play in the mode of action of radiation in living matter. As you will presently see, we shall have to consider not only radiation effects on enzymes themselves but also radiation effects on non-enzymic substances in their relation to enzymes.

Although enzymes are not, in their response to radiation, fundamentally different from other substances capable of reacting with radicals, their inactivation may have more far-reaching biological consequences because of their catalytic properties and the fact that they are present in cells in only small amounts. It has been shown that they are subject to the indirect action of radiation in aqueous solution (Dale, 1940), i.e. via radicals, as well as to direct action in the dry state (Lea, *et al.*, 1944).

When solutions of a crystalline enzyme are irradiated the number of molecules inactivated to a given proportion of remaining activity by a given dose is constant and independent of the initial concentration. In consequence the percentage destruction in a dilute solution is greater than in a concentrated solution, and therefore a dilute solution would appear to be radiosensitive and a concentrated one radioresistant. Thus

radiation doses of the order of 100 r can cause appreciable percentage destruction in a dilute solution (Dale, 1940).

One will have to consider in the discussion whether this dilution effect can be operative in the inhomogeneous interior of a cell. Fig. 1, which refers to the enzyme carboxypeptidase, shows that the efficiency of the radiation decreases only in extremely dilute solutions. This is usually interpreted as being due to the fact that the distance between solute molecules is so great that part of the radicals recombine before they have a chance of reacting with solute.

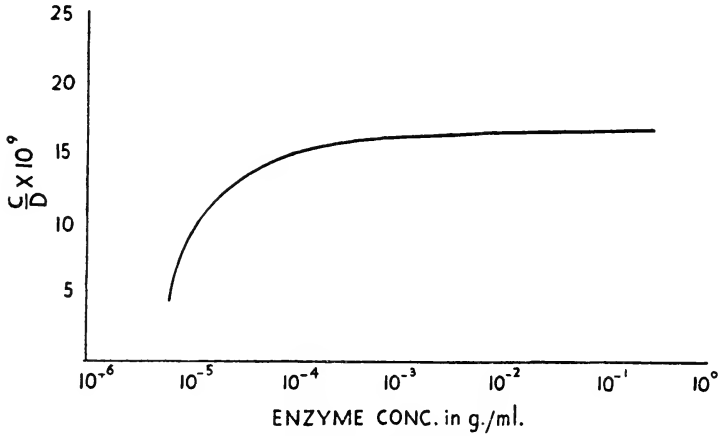


Fig. 1. Yield: concentration relationship for carboxypeptidase.

From a certain concentration onwards, however, the yield remains constant over a wide range of concentrations (Dale, Gray and Meredith, 1949). Other enzymes, e.g. trypsin (McDonald, 1954*b*, 1955) and chymotrypsin (McDonald and Moore, 1955*a*) show some increase in the ionic yield when this concentration is increased. The cause of this is not quite certain though it may be connected with a lower probability of elimination of radicals by the enzyme molecule.

An interesting occurrence of two different ionic yields has been found by Aronson, Mee and Smith (1955), working with α-chymotrypsin, which has an esterase and protease function.

The ionic yield of the esterase activity was three times greater than that of protease activity. A possible explanation given was that there are two active centres in the molecule.

The initial ionic yields for various enzymes generally lie between 1 and 0·1. An example of a very low yield is catalase which is of the order of 10^{-3}, though there is, according to Forssberg (1946), some dose-rate dependence. A low ionic

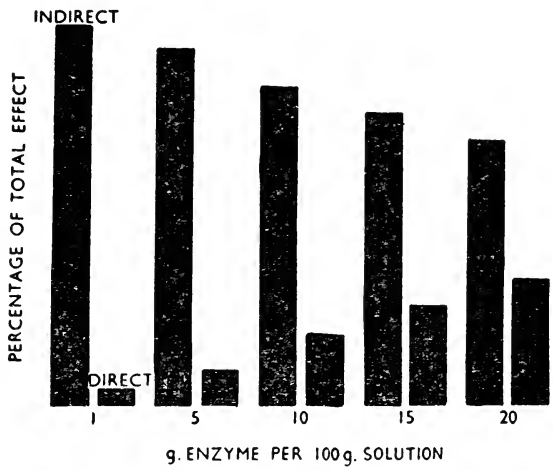


FIG. 2. Relative contribution of "Indirect" and "Direct" action to the total effect of X-rays on carboxypeptidase in solution (Dale, 1947. Reproduced by permission of *Brit. J. Radiol.*).

yield of this powerful enzyme may be of importance to its survival and its consequent availability for decomposing H_2O_2 formed by radiation.

Returning to Fig. 1, it will be seen that the constancy of the yield extends to a concentration of 15 per cent, at which the enzyme is no longer soluble. It will be useful to demonstrate how much of the radiation effect has to be assigned to the direct and to the indirect actions respectively, at the various concentrations.

Fig. 2 demonstrates the respective contributions to the observed effect, at various concentrations, of both the direct

and the indirect actions. Even at a concentration of 20 per cent weight for volume the indirect action is predominant. This presentation applies to enzymes which have a concentration-independent yield of the order of 0·18. If the yields are lower, i.e. if bigger doses are required to achieve the same effect, the chances of the direct action grow correspondingly.

We have now to consider the situation when an enzyme solution is irradiated whilst the solution contains a second solute. Then the available radicals will be shared by both solutes according to their respective concentrations and reactivity, and as a result they will mutually reduce the radiation effect. In other words, the presence of a second solute "protects" the first against the effects of radiation. This protection effect is responsible for an exponential curve when the activity of an irradiated enzyme solution is plotted against radiation dose. As soon as irradiation has started there are two types of solute molecules present, active and inactive, the inactive ones still reacting with radicals, i.e. sharing radicals, though this reaction is not scored. The opposite effect, namely that the presence of a second solute leads to an increase of radiation effects, i.e. sensitization, has so far not been observed with enzymes, though it does occur with other substances. This applies in particular to the presence of oxygen as a second solute, which in many biological radiation responses causes an increase of radiation effects via the HO_2 radicals and H_2O_2. No such increase has been observed with carboxypeptidase (Dale, Gray and Meredith, 1949), ribonuclease (Collinson, Dainton and Holmes, 1950) and trypsin (McDonald, 1954*b*, 1955). Carboxypeptidase (Dale, Gray and Meredith, 1949) and ribonuclease (Collinson, Dainton and Homes, 1950) are stable towards H_2O_2 and are inactivated by the —OH radical. Trypsin is also inactivated by the —OH radical but is reversibly inhibited by H_2O_2 independent of the time of contact, while irradiation has an irreversible effect (McDonald and Moore, 1955*b*).

The protection effect has been shown to operate quite generally. In particular it was shown in the case of D-amino

acid oxidase (Dale, 1942), that the two components making up the enzyme, namely the flavineadenine dinucleotide and the specific protein, when irradiated together lost in terms of oxygen uptake about 60 per cent of their activity, this loss being due to the inactivation of the protein only while the dinucleotide was protected. When irradiated singly, and then joined, the loss was about 90 per cent.

It is important to be aware of possible differences in the response to radiation by non-enzymic substances which may be present as protecting co-solutes, e.g. as substrates for enzymic action or as products of metabolism. The protection effect offers a method by which the effect of radiation on non-enzymic substances can be measured when they are co-solutes in an enzyme solution. The enzymic activity is then the reference against which the effect of radiation on the co-solute is measured. Such measurements make it possible to detect radiation effects when ordinary analytical methods applied to the non-enzymic substance itself would sometimes fail because of the smallness of the effect.

The results of such experiments are as follows (Dale, 1947; Dale, Davies and Meredith, 1949): for large molecules the protective power is roughly proportional to the molecular weight and no specificity is found. However, if one considers small molecules, of which a special atomic group forms the greater part, very marked specificities appear, which, even if they do occur in big molecules, would get lost in the overwhelming excess of other atomic groups of average reactivity with radicals. The outstanding examples of such a specific effect is given by a comparison of the protective effects of urea and thiourea. Whereas urea is hardly protective at all, the substitution of O by S in thiourea causes a 10,000-fold increase in protective power.

Without going into details of the specificity of the protection effect, I should like to stress that sulphur-containing compounds, and sulphur itself, play a special rôle, and that generally the remainder of the molecule has an effect on the protective power of any particular atomic group.

In Table I is listed the protective power of various sulphur-containing compounds, when radiation took place with carboxypeptidase as the indicator. Q_p is the protective power per μg. of protective substance, and Q_s is the protective power of such amounts of protector as contain one μg. of sulphur in each case. One can, therefore, estimate how the non-sulphur residue in any one compound affects the protective power of one μg. of sulphur contained in it, taking the colloidal sulphur as reference. Elemental sulphur is about as protective as thiourea and sodium thiosulphate, but the introduction of

Table I

THE PROTECTIVE POWER OF VARIOUS SULPHUR-CONTAINING COMPOUNDS WITH CARBOXYPEPTIDASE AS THE INDICATOR

	Q_p	Q_s
Thiourea	55	130
Dimethylthiourea	18	58
Colloidal sulphur	110	110
Sodium thiosulphate	24	118

two methyl groups into thiourea causes a considerable decrease in the protective power of the sulphur.

Because of its possible bearing on cell constituents, I should not omit to mention that the straightforward sharing mechanism of radicals between two solutes is not always valid. If it were, the protective power per unit weight of protector would be constant, whereas in certain cases it declines appreciably with increasing concentration of protector. This declining protective power may be of significance from the biological point of view when the effect of protective substances within the interior of cells is considered. The phenomenon, which we called the "changing quotient", is shown in Fig. 3, in which the log of the protective power Q is plotted on the ordinate, and the concentration of the protector on the abcissa. In these experiments carboxypeptidase was used as the indicator.

The diminishing efficiency of the protective substance when its concentration is increased can be explained best by assuming that a protector molecule, after reaction with a radical, may possibly be in a metastable state, or may have formed another organic radical and thus be able to "hand on" the effect of the first collision with a radical to the indicator

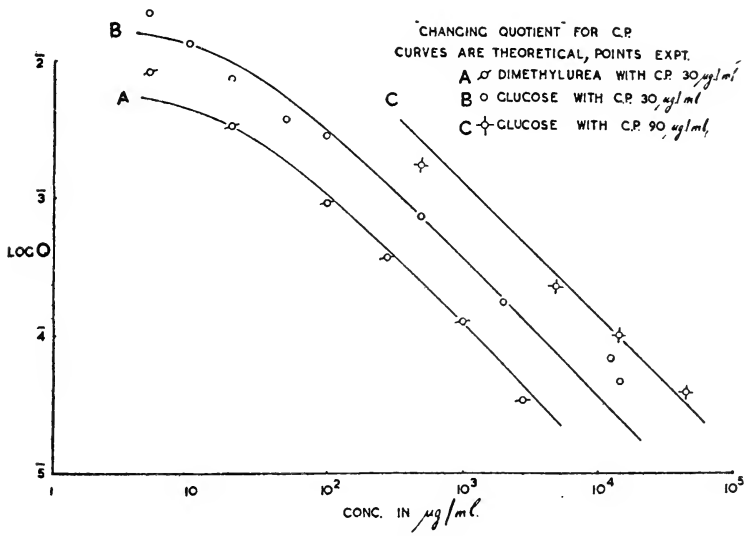


FIG. 3. Curves showing "Changing quotient" for carboxypeptidase. Curves are theoretical, points experimental (Dale, Davies and Meredith, 1949. Reproduced by permission of *Phil. Trans.*).

molecule. Protector molecules reacting in the described manner would fail to fulfil their function as protectors.

Barron and co-workers (Barron and Dickman, 1949; Barron *et al.*, 1949) assume that the principal point of attack by oxidizing radicals is the SH group in enzymes in which SH is essential for enzymic activity. Inactivation could be prevented by blocking the SH groups with mercaptide-forming reagents, and lost activity restored by adding glutathione to reduce the disulphide, provided that the X-ray doses were not so high as to lead to denaturation of the

enzyme protein. When phosphoglyceraldehyde dehydrogenase was inactivated by 100 r, complete reactivation by glutathione was possible, but after 200 r only 62 per cent reactivation occurred. Thus the differentiation between action on SH groups and denaturation of protein lies within rather narrow limits and and does not seem fully justified.

Closely linked with the question of denaturation of protein is the observation of after-effects. Continued inactivation after cessation of irradiation has been found with pepsin (Anderson, 1954), and with trypsin (McDonald, 1954*a*). Some modification of the enzyme molecule has been produced which makes the molecule more sensitive to thermal denaturation, similar to the action of radiation on the albumin examined by Fricke (1952).

Effect of α-radiation. With regard to the effect of radiation of different ionization density, only one extensive study on the effect of α-radiation as compared with X-radiation, on carboxypeptidase, has been carried out (Dale, Gray and Meredith, 1949). The result was that the efficiency of α-rays was shown to be only one twentieth that of X-rays, and this low efficiency could be accounted for by the δ-radiation which accompanies the α-radiation, and is similar in ion density to X-radiation.

We have so far dealt with enzymes and co-solutes in solution. There is, however, evidence that there are many enzymes firmly bound to cell structure. Not very much is known about these from *in vitro* experiments. Mazia and Blumenthal (1950) made an attempt to investigate a monomolecular film of pepsin-albumin on the surface of water, and exposed it to radiation. This difficult experiment is open to some criticism since the substrate, which was not in great excess of the enzyme, was also exposed to radiation. However, they reported an inactivation of a thousand enzyme molecules per ionization under the circumstances of the experiment. More model experiments with phase-bound enzymes would be valuable.

In conclusion of this survey of *in vitro* experiments with enzymes it may be useful to stress the main features of the

mode of action of radiation which will have to be considered when attempting to correlate the results with the structural organization and the metabolic activities of cells.

(1) Can one assume that the dilution effect may operate inside a cell? In other words, are there intermicellar spaces through which enzyme molecules diffuse from storage depôts when called upon by metabolic requirements of the cell?

(2) Are these intermicellar spaces in the inhomogeneous cell structure filled with high concentrations of protective substances?

(3) Is there a spatial separation between substrates and surface-bound enzymes which may involve an action of radiation on substrates in transit?

(4) Should one consider not a depletion of stored enzymes but rather a decrease of the functional fraction of enzymes which, by slowing down reaction velocities and possibly also by the initiation of non-enzymic chain reactions, disorganizes the delicate sequence of metabolic steps?

(5) Can one expect from an analysis of tissue extracts or homogenates irradiated as such, or made from irradiated tissues, any answer to the question of the participation of enzymes in biological radiation effects?

Answers to all these questions will depend on the degree of knowledge of the internal organization of cells at the submicroscopic level, and I hope that the discussion will clarify some of the issues raised.

REFERENCES

ANDERSON, R. S. (1954). *Brit. J. Radiol.*, **27**, 56.
ARONSON, D., MEE, L., and SMITH, C. L. (1955). IV Int. Conf. Radiobiol. Edinburgh: Oliver & Boyd.
BARRON, E. S. G., and DICKMAN, S. (1949). *J. gen. Physiol.*, **32**, 595.
BARRON, E. S. G., DICKMAN, S., MUNTZ, J. A., and SINGER, T. P. (1949). *J. gen. Physiol.*, **32**, 537.
COLLINSON, E., DAINTON, F. S., and HOLMES, B. (1950). *Nature, Lond.*, **165**, 266.
DALE, W. M. (1940). *Biochem. J.*, **34**, 1367.
DALE, W. M. (1942). *Biochem. J.*, **36**, 80.
DALE, W. M. (1947). *Brit. J. Radiol.*, Suppl., **1**, 46.

DALE, W. M., DAVIES, J. V., and MEREDITH, W. J. (1949). *Brit. J. Cancer*, **3**, 31.
DALE, W. M., GRAY, L. H., and MEREDITH, W. J. (1949). *Phil. Trans.*, **242A**, 33.
FORSSBERG, A. (1946). *Acta radiol., Stockh.*, **27**, 281.
FRICKE, H. (1952). *J. phys. Chem.*, **56**, 789.
LEA, D., SMITH, K. M., HOLMES, B., and MARKHAM, R. (1944). *Parasitology*, **36**, 110.
MAZIA, D., and BLUMENTHAL, G. J. (1950). *J. cell. comp. Physiol.*, **35**, 171.
MCDONALD, M. R. (1954*a*). *Brit. J. Radiol.*, **27**, 62.
MCDONALD, M. R. (1954*b*). *J. gen. Physiol.*, **38**, 93.
MCDONALD, M. R. (1955). *J. gen. Physiol.*, **38**, 581.
MCDONALD, M. R., and MOORE, E. C. (1955*a*). *Radiation Res.*, **3**, 38.
MCDONALD, M. R., and MOORE, E. C. (1955*b*). *Radiation Res.*, **2**, 426.

DISCUSSION

Alexander: I think we are extremely fortunate that in the days when the indirect effect was not well understood Dr. Dale happened to choose the particular enzyme systems which he has described, because this enabled him to put the indirect effect on the sound basis which we now take for granted. If he had used other proteins or, as criteria, changes other than inactivation of enzymes, then the effect of concentration and the relation with dose would have been much more complicated. When solutions of protein—we have studied serum albumin and lysozyme—are irradiated in dilute solutions, aggregation occurs and units of very much larger molecular weight are formed. There is no simple relationship between the dose or protein concentration and size of the aggregates formed. This often exceeds several million and with higher doses the whole material becomes quite insoluble. All these molecules which form part of the very large aggregate have, in a sense, been removed from the bulk solution, but are not necessarily enzymatically inactive. Depending on the method of test, very strange dose-relationships between radiation effect and/or concentration may be observed. Changes in ionic strength can alter critically the aggregation phenomena and thus influence the radiation effect in a way which cannot be explained from simple consideration of indirect action.

The second point which I want to make is that for these aggregation phenomena direct action is remarkably efficient, i.e. if we irradiate serum albumin dry, and then dissolve it up and measure the amount of change which has occurred, we find that direct action is remarkably great. Six electron volts (or a G value of 18) is sufficient for the disappearance of a protein molecule and its shift into an aggregate; if we were choosing this as a criterion, we would find on Dr. Dale's histogram, giving the proportion of direct and indirect effect at different concentrations (*see* page 27), that the direct effect would be the most important at the local concentrations of proteins encountered in cells.

My last point concerns the attack on the tyrosine in proteins. When

serum albumin has been irradiated in dilute solution, the u.v. absorption peak at 2,800 increases and this has been interpreted by Barron and others as a reaction by the free radicals with the tyrosine residues in the protein molecule. In reality this increase is due to aggregation and not due to a change in the actual light-absorbing groups. On irradiation the protein molecules form aggregates which scatter more light. The amount of light scattered varies inversely as to the fourth power of the wavelength, and a solution which does not appear cloudy in visible light may scatter a great deal at 2,800 Å. We proved that the increase in absorption at the 2,800 peak is entirely due to aggregate formation, by measuring the amount of light scattered in a special instrument. In the case of lysozyme which contains much tryptophan, the position is slightly different: one does first of all get a decrease in the absorption peak and this is due to the destruction of the tryptophan. With higher doses the absorption goes up, but this increase is due to aggregation.

Dale: I should like to answer the first part. I have no experience of the second part, which is actually a communication of your experiments rather than a question. I think it would be rather unfortunate to choose this criterion of aggregation, because it is a very common experience with all colloids that the particle size increases on standing. I should like to ask whether you have examined these solutions after irradiation has been finished, and whether there are after-effects of aggregation or not.

Alexander: Not after the first 30 minutes, which was the shortest period in which we were able to look at it after irradiation.

Dale: When you precipitate colloids with various precipitins, with various salts, you find continuous aggregation leading eventually to flocculation. You must have had a similar phenomenon because you mentioned the effect of addition of salts. I don't know whether you varied valency of the salts using divalent ions and trivalent ions and so on and whether you had a negative or a positive colloid, but the very fact, as you say, that the enzymatic activity is not necessarily changed does not bring this phenomenon within these experiments, because what we measured is the effect on the activity of the enzyme rather than on aggregation. I think the aggregations are rather non-specific changes which with bigger doses probably also point to denaturation and I think it does not affect activity measurements with radiation, which are strictly quantitatively what you would expect, that doubling the dose or making the dose 100 times as great has 100 times greater effect, apart from the region where you have wide separation of solute molecules with recombination of radicals.

Forssberg: When speaking about colloids and irradiation it may be relevant to recall that J. A. Crowther and others, some twenty years ago, studied the changes produced by very small doses on, for example, colloidal gold and graphite, but also on proteins. It would seem that irradiation causes cyclic changes both in particle size and in charge. These changes sometimes proceed even after the irradiation is finished, which implies that they are a function of the time of assay. It is not known whether similar effects occur *in vivo*.

Alexander: I think we should differentiate between lyophobic colloids, which are essentially unstable colloids which will aggregate in time, and solutions of macromolecules with which one deals in serum albumin which is stable. The reason why I mentioned the physical changes in proteins produced by radiation is that they can play a part in removing the enzyme from its sphere of action and this may be as serious to the cell as true inactivation. Aggregation induced by radiation is very dependent on the conditions of irradiation and may contribute to the variation in the radiosensitivity of cells with changing conditions.

Dale: This is a useful suggestion.

Popjak: I would like to raise a question about our general way of thinking about effects of radiation on enzymes. I suppose the reason why most people are looking for inactivation of enzymes really springs from the overall effects observed, i.e. that the radiation eventually kills an animal or a cell. Now, are we right in assuming that radiation will necessarily inactivate an enzyme? The biological effects that are observed are observed with relatively small doses; how far is one justified in concluding from the irradiation *in vitro* with very large doses of a crystalline enzyme, divorced from its substrates and all its other companions, that the same sort of phenomenon is operating in the cell? When an enzyme is inside the cell it is working fairly fast, and there is a continuous movement of electrons and protons in and around the molecule probably forming some kind of resonating system. I wonder whether we might not by irradiation change enzyme specificity, change rates of reactions, and whether it might not be worth while directing some work towards that end rather than watching the enzyme inactivation, and whether more information as to biological effects might not be obtained in this way.

Dale: This is just what I had in mind when I put those questions at the end of my presentation. My point of view is that it is quite possible that a very minute functional part is changed while it is in transit and that small changes may upset the proper sequence of events, changes which may be so small that they are not analytically detectable, but whilst the reaction is going on in the cell it may be of much greater significance than the depletion of absolute amounts of enzyme. If, for instance, in the enzymic reaction the functional part is slowed down it cannot provide precursors for another reaction at the right time, so that the integration of various interdependent reactions is destroyed and, in my opinion, any attempt at trying to find a difference in enzyme content of irradiated tissues or cells or disintegrated cells is, from the start, completely futile because you only catch the total amount of enzyme which does not matter at all. We are quite agreed that radiation can only deal with a minute fraction of the enzyme present, but it all depends on whether this minute fraction in the circumstances in which it works in the cell is not relevant to the effect and, of course, from the practical point of view it is very difficult to find experimental conditions for checking what you suggested.

Butler: I would like to support Popjak's view on these grounds: you have two classes of enzymes, those that are present in the cell in quite

large quantities in which case, under any reasonable circumstances, the percentage of inactivation is quite small, whether your reaction is direct or indirect. The other case is the possibility of enzymes being present in very small amount; as McIlwain has shown (1946, *Nature, Lond.*, **158**, 898), the possibility exists of enzymes being present only to the extent of a few molecules per cell. But the situation is also difficult there because the chance of a unique molecule of an enzyme being inactivated is correspondingly small. So that in either case it seems to me that the possibility of an enzymic explanation of metabolic effects is not a very favourable one.

THE ACTIVITY OF ENZYMES AND COENZYMES IN IRRADIATED TISSUES

ANTOINETTE PIRIE

Nuffield Laboratory of Ophthalmology, University of Oxford

THE biochemical effects of radiation have been recently reviewed by Ord and Stocken (1953), DuBois and Petersen (1954), Errera (1955) and by Bacq and Alexander (1955) and these valuable reviews form the basis from which any survey of this jungle of a subject must be made. They make a general survey of the jungle unnecessary and I propose to define the problem as best I can and then to describe in some detail those pieces of experimental work which provide evidence for an initial effect of radiation on enzymes in the cell. The effect of radiation on DNA, RNA, protein and phosphorus metabolism is being considered by other speakers and will not be touched upon.

If we are looking for an effect of radiation on cell enzymes, what characteristics should we demand? A change in enzyme activity found after radiation of the whole animal could be a direct or indirect effect, i.e. reaction with the ionizing radiation or particle itself or with the radicals formed in the medium. It could also be a secondary effect resulting from structural or chemical changes in other molecules which repercuss on the activity of the particular enzyme we are studying. It seems probable that such secondary effects on enzymes will become more marked with time unless recovery sets in. Both direct and indirect effects will be instantaneous and may be detectable as soon as it is possible to test; but they may or may not be maximal at this time. An enzyme, slightly damaged by direct or indirect action, may continue to decay. Thus, for example, Anderson (1954) found that pepsin, and McDonald (1954) found that trypsin gradually lose further activity dur-

ing the 24 hours following X-radiation. Kleczkowski (1954) has found that if chymotrypsin is irradiated with ultraviolet light and then kept at 2°, no further activity is lost, but if kept at 37° for 48 hours the irradiated sample loses more activity than a parallel control sample. Irradiation has made the enzyme abnormally susceptible to body temperature. We cannot therefore draw any hard and fast line between the cause of enzyme change detected as soon after radiation as we are able to make the estimation, and enzyme change that develops gradually.

The time that must elapse between irradiation and biochemical testing is necessarily long compared to the time taken in metabolic reactions; a 10-second illumination of green algae in the presence of carbon dioxide is sufficient for the synthesis of a very large number of compounds (Bassham *et al.*, 1954). The time that elapses between irradiation and metabolic examination is ample for a long sequence of changes to interpose between direct and indirect effects of radiation and the change we measure.

It does, however, seem worth while to concentrate on what Errera (1955) has termed initial effects. The fact that few, if any, such effects have been found may be an indication of our ignorance of large parts of tissue metabolism, and the fact that even the known parts have not yet been thoroughly surveyed in relation to radiation. But it could also be explained if the only effect of radiation is to upset the molecules of DNA and to upset its synthesis. This may well be an enzymic effect since one could expect that DNA molecules may have enzymic activities and will be synthesized by enzymic processes, but at least it restricts the area of investigation. I do not think any evidence we have at present can decide. Certainly radiation affects cells other than those capable of division. Patt (1955) has pointed out that there are many departures from the simple condition relating radiosensitivity to growth and differentiation. The metabolic activity of the cell can determine radiosensitivity, e.g. chilling lymphocytes to 2° either shortly before or shortly

after radiation will remove the protective effects of anoxia or of cysteine treatment (Patt, 1955). A low temperature also seems to make the glycolytic enzyme systems of tumour and retina more sensitive.

One concept of the reason why it is difficult to find an immediate change in enzymic activity is that radiation has a greater effect on enzyme synthesis than on the enzymes themselves. The cell, therefore, may only gradually go down hill as enzymes fail to be replaced; but in yeast and *Escherichia coli*, which have been investigated, the ability to form adaptive enzymes seems unimpaired immediately after a dose of X-rays that has killed 90–99 per cent of the cells. Thus Brandt, Foreman and Swenson (1951) find that yeast cultures given 4,800 r, which kills 90 per cent of the cells, will still form galactozymase. Spiegelman, Baron and Quastler (1951) found both galactozymase and maltozymase formation unaffected. Yanofsky (1953) found that *Esch. coli* could still form lactase normally after a dose of 5,000–10,000 r and Billen and Lichstein (1952) showed that hydrogenlyase formation by *Esch. coli* was normal after 15,000 r, for 100 minutes, although it then fell off.

Concerning the effect of radiation on enzyme formation in animal tissues Rauch and Stenstrom (1952) found that 400–600 r of X-radiation restricted to the pancreas, in dogs with pancreatic fistulae, caused a lowered secretion of amylase, trypsin and lipase when tests were made 12 hours later. The volume and pH of the secretion remained unchanged but the enzyme content fell. The effect was reversible but could be reproduced with further irradiation. The glands showed no histological damage. Since the secretion of enzymes was not studied at once after radiation we cannot say that this is an initial effect, but it is an interesting approach.

Therefore, as there is doubt in this last case, we can say that irradiation with X-rays [ultraviolet does inhibit (Errera, 1955)] has no immediate effect on enzyme synthesis. Thus, a bacterial cell can continue to maintain its integrity and to synthesize some at least of its enzymes after an amount of

radiation that prevents cell division. This does not lend support to the view that the gradual decay of enzymes after irradiation of animals is due to failure of synthesis, but the evidence is far too scanty to be taken as refuting such a view.

It seems useless to attempt to catalogue all the work that has been done which shows delayed effects of radiation on cell enzymes. This includes my own work on lens metabolism. It would appear to be more profitable to make a few bald statements which can be amplified, if necessary, in discussion. This may lead to oversimplification but a detailed presentation leads only to confusion.

Within the boundaries of our ignorance it seems true to say that lethal doses of radiation do not immediately change the overall oxygen consumption of the animal or the major respiratory enzymes. There is no change in oxygen consumption of guinea pigs after an LD_{50} of X-rays (Smith, Buddington and Greenan, 1952) or of the rat during the first four days after a lethal dose (Mole, 1953). On the other hand, liver dispersions examined 6 hours after whole body X-ray show increased endogenous respiration (Kunkel and Phillips, 1952), as do also bone marrow cells from the rabbit examined as soon as possible after radiation (Altman, Richmond and Solomon, 1951). These increases may indicate that there has been a change in substrate concentration or another form of enzymic activation. They are evidently insufficient to alter the oxygen consumption of the whole animal. With *Esch. coli*, Billen, Stapleton and Hollaender (1953) found that with one strain a killing dose made no immediate difference to respiration with glucose, succinate and pyruvate. With another there was an immediate fall in oxygen uptake with pyruvate.

Cytochrome oxidase and succinic dehydrogenase, two major respiratory enzymes of the mitrochondria, have been widely investigated and in no case has any change been found as a result of radiation, even hours or days later (Powell and Pollard, 1955; Ashwell and Hickman, 1952; LeMay, 1951; Hagen, 1955; Ryser, Aebi and Zuppinger, 1954; Thomson, Tourtellotte and Carttar, 1952; Fischer, Coulter and Costello,

1953). Cytochrome *c* itself is not affected in *Esch. coli* (Hagen, 1955), nor is the uptake of radioactive ^{59}Fe into cytochrome *b* of the liver (Bonnichsen and Hevesy, 1955).

The citric acid cycle accounts for a large part of the oxidative metabolism of the tissues. The activity of the enzymes of this system can be determined by determining the formation of fluorocitric acid after injection of fluoroacetate. DuBois, Cochran and Doull (1951) have found that a lethal dose of X-rays to the rat inhibits fluorocitric acid accumulation in the spleen, thymus and kidney within three hours. A dose of 100 r inhibits synthesis temporarily in the spleen only. Now within three hours these tissues will be already grossly altered structurally and hence one cannot conclude that radiation has had a direct or indirect effect on the enzymes concerned. That it is a secondary effect is suggested by the fact that if the spleen is exteriorized and it alone is irradiated (Table I) the fall in citric acid synthesis does not occur (Petersen, Fitch and DuBois, 1955).

Table I

CITRATE SYNTHESIS IN RAT SPLEEN

	Control	*24 hours after 800 r*	
		To whole body	*To spleen only*
Citric acid μg./g.	1001	676	1002
DNAP mg./100 g.	124	55	98

Next, to consider coenzymes. These substances are ubiquitous and function in many different metabolic processes so that change in activity could have wide effects. Diphosphopyridine nucleotide coenzymes are thought to be synthesized in the nucleus and change might indicate change of nuclear function. No immediate change in their activity has, however, been found. Eichel and Spirtes (1955) found no change in the oxidized or reduced DPN content of rat liver 1·5 min. after a lethal dose to the whole animal. Coenzyme A activity, measured as the power of the animal to acetylate sulphanil-

amide (DuBois, Cotter and Petersen, 1955) or *p*-aminobenzoic acid (Thomson and Mikuta, 1954) or to form hippuric acid (Schrier, Altman and Hempelmann, 1954) was unimpaired. It therefore appears that both coenzyme A and the enzymes concerned with these acetylations function normally. The level of coenzyme A and of nicotinic acid, which is some measure of DPN and TPN, remained normal in the early stages of X-ray cataract (van Heyningen, Pirie and Boag, 1954). The level of pyridoxin in the liver remains unaltered (MacFarland *et al.*, 1950). Glutathione has not been found to decrease in any tissue immediately after radiation (Bacq and Alexander, 1955).

The glycolytic activity of tissues has not been extensively studied. One particular investigation will be described later but here one can say that where individual enzymes concerned in glycolysis have been examined no change has been found immediately after radiation. Thus aldolase, glyceraldehyde phosphate dehydrogenase and lactic acid dehydrogenase of lens, liver, kidney and spleen are not early affected (van Heyningen, Pirie and Boag, 1954; DuBois and Petersen 1954).

Throughout, one has been expecting a fall in enzyme activity; but results show that some enzyme processes are immediately increased. Altman, Richmond and Solomon (1951) showed that if the bone marrow was removed from rabbits immediately after giving 800 r to the whole animal and the synthesis of saturated and unsaturated fatty acids from labelled acetate was measured *in vitro* the irradiated bone marrow had 2–3 times the activity of the normal (Table II). The oxygen uptake by the tissue was also greater. The actual

Table II

EFFECT OF RADIATION ON THE SYNTHESIS OF FATTY ACIDS AND ON RESPIRATION OF THE BONE MARROW OF THE RABBIT

	Fatty acids (% *pre-radiation value*)		O_2 *uptake* μl. O_2/*g. wet wt. in 3 hours*
	Saturated	*Unsaturated*	
No radiation	100	100	300
0 hours	231	344	810
48 hours	108	—	280

time that elapsed between irradiation and the measurement of synthesis is unfortunately not given. Later, the bone marrow decayed. We know that coenzyme A, the coenzyme concerned in fatty acid formation, is normal after radiation and it appears that the enzymes are activated at once, either directly or by change in substrate concentration, or change in permeability of the mitochondria which are the seat of fatty acid synthesis.

This ties in with the work of Hevesy and Forssberg (1951) who found that if mice given 2,000 r were then injected immediately with ^{14}C-labelled glucose, and the exhaled CO_2 collected, starting 8 minutes later, the irradiated mice gave off less CO_2 over the first hour than did the controls. There was also increased ^{14}C in liver fats under these conditions.

The synthesis of haemoglobin also appears to be stimulated immediately. Richmond, Altman and Solomon (1951) again using the rabbit and a whole body dose of 800 r, found that bone marrow and spleen dispersions taken immediately after radiation incorporated ^{14}C-labelled glycine into haemin and globin with greater rapidity than the normal. Forty-eight hours after radiation synthesis had declined.

Similarly, Nizet, Lambert and Bacq (1954) found stimulation of haemin synthesis *in vitro* by reticulocytes taken from a dog 30 minutes after a whole body dose of 500 r or by reticulocytes irradiated *in vitro*. In three of four dogs tested it appeared that plasma from the irradiated dog stimulated haemin formation in non-irradiated cells. This makes it appear that stimulaation of synthesis is not a direct or indirect effect on enzymes of the red cell but a change in environment.

On the other hand, Bonnichsen and Hevesy (1955), who point out that "Haemoglobin is one of the comparatively few molecular constituents of the adult organism that is formed in close connection with cell division", found decreased haemoglobin formation in irradiated guinea pigs which were injected with ^{59}Fe 6 hours after X-ray and killed 17 hours later. This decrease in synthesis did not take place immediately after radiation and it is suggested that the red blood corpuscles of the marrow which are in an advanced stage of maturation

complete the synthesis of their haemoglobin after a dose of radiation which will ultimately stop new haemoglobin formation entirely.

However interesting may be these results which show immediate stimulation of fatty acid synthesis and of haemin and globin formation, there is always the doubt that they are direct or indirect effects of radiation since they have been obtained with tissue preparations from animals which had received whole body radiation. Therefore the effects might be secondary and due to environmental change, i.e. change in available substrates in the tissues. *In vitro* work with such enzyme systems might give the answer.

To turn now to experiments where rather simpler conditions have been used than irradiation of the whole body:

Formation of adrenal steroids

Ungar and co-workers (1955) have found that irradiation of the perfused adrenal gland of the calf reduces the formation of adrenal steroids. These glands, obtained from the slaughterhouse, are perfused and irradiated simultaneously for 2–3 hours, the dose being around 2,000 r of gamma-radiation from ^{60}Co. The blood, to which ACTH was added to stimulate steroid formation, is passed through the gland only once and therefore received only a small dose of radiation. At the end of the perfusion the adrenal steroids in the blood were isolated and estimated chromatographically. Production of hydrocortisone, corticosterone and unidentified steroids was markedly diminished.

Rosenfeld and co-workers (1955) further showed that if various steroid precursors were added to the perfusing blood, irradiation inhibited their conversion.

Table III shows that 11β-, 17α- and 21-hydroxylations are inhibited as well as oxidation of the Δ^5-3β-hydroxyl group to to Δ^4-3-ketone group. The percentage decrease in the conversion products paralleled both in range and variability the percentage decrease in corticoid output which was found in

Table III

INHIBITION OF STEROID SYNTHESIS IN PERFUSED ADRENAL GLAND BY GAMMA-RADIATION

Dose 2,000–3,000 r

Substrate		*Transformation product*	*Decreased conversion per cent*	*Specific reaction inhibited*
Substance S	→	Hydrocortisone	75, 34, 54	11β-hydroxylation
DOC	→	Corticosterone	35, 55	11β-hydroxylation
Progesterone	→	Hydrocortisone	37 (+7)	11β, 17α + 21-hydroxylation
21-Desoxy-cortisone	→	Cortisone	22, 19	21-hydroxylation
Δ^5-Androstene-3β-OH-17-one	→	11β-OH-Δ^4-androstene-3 : 17-dione	48, 21	Δ^5-3β-OH-Δ^4-3-ketone

the ACTH-stimulation studies. Radiation therefore appears to inhibit many of the steroid-synthesizing enzymes and to have little specificity. The inhibition is apparent immediately radiation ceases though it is true that the time of radiation is considerable as is also the dose. However, other systems *in vitro* are far more resistant to radiation than this. A point of interest is that the enzyme which catalyses 11β-hydroxylations is active in mitochondrial preparations (Brownie and Grant, 1956) and the effect of radiation can be tested therefore on this isolated enzyme that seems to be radiosensitive in the tissue.

A further point is to try to relate the changes in the gland *in vitro* with changes *in vivo* after radiation. Radiation of the rat with 800 r causes loss of ascorbic acid and of cholesterol from the adrenal within 1 hour (Bacq and Alexander, 1955). Hochman, Bloch and Frankenthal (1953) found that 25–50 r caused decrease in adrenal ascorbic acid tested 1 hour after irradiation. The decrease, however, was not found to be dose dependent; large doses still caused only about 26 per cent decrease of adrenal ascorbic acid. Prof. Pincus tells me that the ascorbic acid content of the adrenal glands used in his perfusion work was extremely low and therefore no studies of change during irradiation were made as it was felt these would

be meaningless. Excretion of steroids after radiation seems to have been little studied. It is therefore impossible at present to link the *in vitro* depression of steroid synthesis with *in vivo* changes.

Effect of radiation on the retina

Crabtree (1936) found that if he gave long periods of irradiation with radium—he does not specify the dose—either to tumour tissue or to retinal tissue *in vitro* at 0°, the anaerobic glycolysis was very much depressed whereas the respiration of the tissue was unaffected. Tumour irradiated at 37° showed no failure of anaerobic glycolysis. Retina could not be irradiated at the higher temperature as the control non-irradiated retina was unstable here.

Crabtree and Gray (1939) repeated this work using known doses of X-rays, beta-rays or gamma-rays, keeping the time of irradiation short and measuring the metabolism as quickly as possible after radiation ended. They studied the retina of the rat. Table IV shows that after a dose of 1,250 r given in

Table IV

INHIBITION OF ANAEROBIC GLYCOLYSIS OF RAT RETINA BY X-IRRADIATION AT 0–5°

Anaerobic glycolysis was measured 20 minutes after end of radiation

Time radiation minutes	*Dose r*	*Average per cent inhibition of anaerobic glycolysis*	
		10 *minutes*	20 *minutes*
5	1250	13·0	15·3
10	2500	41·0	42·0
20	5000	61·8	62·5
40	10000	67·0	71·8

5 minutes at 0° the anaerobic glycolysis, when measured 20 minutes later, had fallen by 13 per cent. As the dose of radiation given was increased the inhibition of glycolysis became more marked. Fig. 1 shows that equal doses of β-radiation and of X-radiation have the same effect. A criticism of this work is that the figures given are percentage decreases and if one calculates back from these percentages to the actual

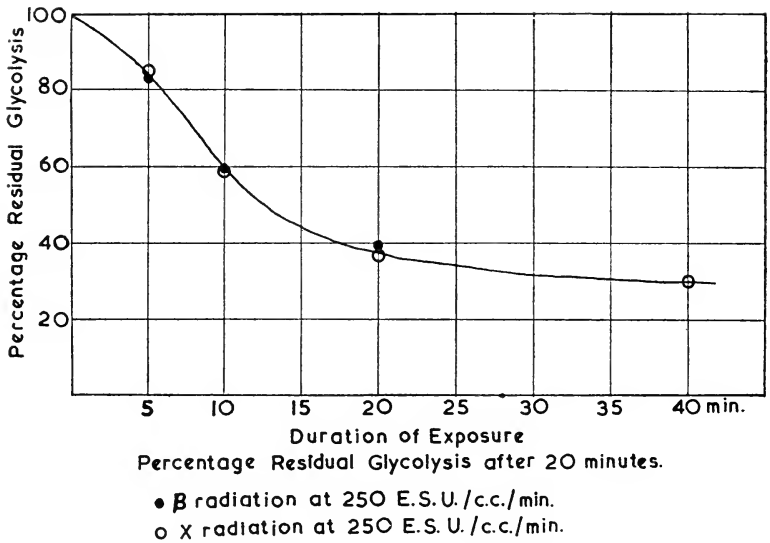


FIG. 1. Equivalence of β-rays and X-rays in depressing anaerobic glycolysis of rat retina.

manometric readings that lie behind them then it seems difficult to place much confidence in the small change found after the lowest dose of radiation in the first 10 minutes of experiment. But this figure does not stand by itself. It forms part of a series of results all of which show that radiation inhibits anaerobic glycolysis when this is measured as soon as possible after radiation ceases. Table V gives some results from an

Table V

RESPIRATION AND GLYCOLYSIS OF TISSUE AFTER IRRADIATION AT LOW OR BODY TEMPERATURE

Tissue	*Time of irradiation T_r* hours	*Temp.* °C	Q_{O_2}			$Q_M^{N_2}$		
			T_o	T_r	*Irradiated*	T_o	T_r	*Irradiated*
J.R.S.	4	0–5	8·2	7·0	6·8	34·3	35·6	8·6
	4	37·5	—	—	—	41·2	42·0	36·0
Retina	2	0–5	—	20·9	18·1	—	77·1	4·5, 9·4
	1	0–5	—	19·4	17·3	—	68·4	25·6→5·8

earlier paper where the actual Q_{O_2} and $Q_M^{N_2}$ (anaerobic glycolysis) are given and show the great difference in result according to temperature. Therefore, I consider that we have here a case where radiation——a relatively large but not enormous dose—inhibits an enzyme sequence *in vitro*. If the retina is like the tumour used in Crabtree's earlier work it appears that irradiation must be carried out at 0° for the effect to occur.

No work, as far as I know, has been published on the effect of radiation on retinal metabolism *in vivo*. But that is not to say that radiation has no effect. First of all, it is known that low doses of X-rays produce a sensation of light—the X-ray phosphene has been known since the last century—and there is considerable evidence that X-rays act on visual purple, the light-sensitive substance of retinal rod cells (Lipetz, 1955*a* and *b*). This action of X-rays in stimulating the retina is not known to have any relation to the inhibitory action of X-rays on retinal glycolysis as the visual purple in rat retinas used for metabolic experiments may have been largely bleached although the retinas were prepared carefully in dim light. Let us just use this as an indication that X-rays can stimulate retinal tissue *in vivo*.

Evidence that X-rays have a very rapid damaging effect on the retina *in vivo* has come from recent work by Cibis, Noell and Eichel (1955) who have found that a dose of 2,000 r given to the eye of a rabbit abolishes the *b*-wave of the electroretinogram within 10 minutes of the cessation of radiation. This change may be reversible but with larger doses the rod cells of the retina show degenerative changes within 3–5 hours, and over a period of days these cells disappear. The changes produced by X-rays are strikingly similar to those produced by injection of iodoacetic acid, that well known inhibitor of the enzymes of glycolysis. Noell (1951) found that non-lethal doses of iodoacetate caused immediate reduction in amplitude of the electroretinogram measured in the living animal and gradual histological decay of the rod cells. Cone cells were relatively immune, as they are also to X-rays.

Various considerations come to mind. First, is the rapid diminution of the electroretinogram due to bleaching of the visual purple by the X-rays? This seems unlikely since Lipetz (1955*b*) using isolated retinas found little bleaching until 10^5–10^6 r were given and then the picture was muddled by heating effects. Calculations based on ratio of threshold dose to bleaching dose for light and for X-rays also predict that a very large dose of X-rays is necessary for bleaching. Second, is the change in electroretinogram and the degeneration of the rod cells due to vascular change in the choroid? Cibis, Noell and Eichel (1955) state that vascular engorgement occurs, but it is said that this type of retinal degeneration would not be expected from such choroidal change.

With a dose of 1,400 r of X-rays we have noticed degenerative changes in the outer limbs of rod cells of the rabbit eye when the animal was killed some weeks or months later. We have not examined many animals. Biegel (1955) using radiation from the betatron failed to find more than minimal changes after 3,600–4,500 r, in the rabbit retina. The results, therefore, are a little variable.

In trying to assess the effects of radiation on the retina let us return to the work of Crabtree and Gray. At 0° the anaerobic glycolysis was inhibited. Now at 0° Terner, Eggleston and Krebs (1950) have shown that retinal tissue is unable to maintain osmotic control. It loses K and takes up Na. Low temperatures have, in fact, a most remarkable effect on tissues. It has long been known that they swell at this temperature, and Conway, Geoghegan and McCormack (1955) find that kidney and muscle tissue frozen in liquid O_2, ground up and then maintained at 0°, lose ATP and hexosephosphate and increase their non-protein N. Hence, if one irradiates a tissue at 0° not only is it in a state of metabolic arrest but it will be in a state of metabolic decline. The change in K and Na in the retina at 0° are reversible at 37° in the presence of glucose and glutamate but not in their absence.

It seems possible that radiation could have a quite different effect at low temperatures from that at normal—without

having recourse to an oxygen effect to explain this. This problem needs a great deal more work but seems an example of a rapid effect of X-rays on an enzyme system containing at least 11 enzymes. No individual enzyme was studied, but there is a certain specificity since the respiratory mechanism is unaltered.

Auxin

A third instance where radiation is thought to have a direct or indirect effect on a cytoplasmic enzyme is the inhibition of auxin synthesis in the plant by very low doses of X-rays. Skoog (1935) first investigated the effect of radiation on auxin and thought that auxin itself was inactivated rather than that its synthesis was affected. Later, Gordon and Weber (1955) have concluded that auxin is not particularly sensitive to radiation, but that the synthesis is easily inhibited (Weber and Gordon, 1951*a*, *b* and *c*; 1952*a*, *b* and *c*; Gordon, 1956). The effect was first described by Weber and Gordon (1951*a*) in an Argonne National Laboratory report. Since that time there have been many brief reports in ANL publications and Gordon gave a brief paper at Cambridge in 1955, but, as far as I am aware, no details of the methods used and the results obtained have ever been published and though one can get some notion of the methods from others of Gordon's papers not concerned with radiation, I have yet found it difficult to assess the work. I feel a protest must be made against this habit of publication of brief reports in laboratory journals which are not available to most scientists. The subject deserves more serious treatment.

Now, to summarize as critically as possible the ANL reports on the inhibition of auxin synthesis by X-rays. Weber and Gordon (1951*a*) find, first, that low doses of X-rays cause an immediate drop in the auxin content of the young mung bean plant; second (Weber and Gordon 1951*b*), that shoots of the mung bean infiltrated with tryptophan, and then irradiated, form less auxin than similar non-irradiated shoots; third

(Weber and Gordon 1952*a*), extracts of irradiated mung bean plants form less auxin from tryptophan than extracts from normal plants. These changes were noticeable immediately after radiation with low doses such as 10–50 r but synthesis of auxin was not stopped completely even by very high doses.

The synthesis of auxin from tryptophan goes through a series of steps. Weber and Gordon (1951*c*) found that immediately after irradiation of the plant with 25–50 r there was a rise in concentration of an aldehyde. Similarly, using extracted enzymes from irradiated plants Weber and Gordon (1952*a*) found increased formation of an aldehyde which is considered to be indoleacetaldehyde. They therefore conclude that it is the enzyme which converts indoleacetaldehyde to indoleacetic acid or auxin that is specifically inhibited by radiation.

If there is any doubt about this—and until details of the work done between 1949 and 1955 have been published there must be a doubt—I think it lies in the conclusion that irradiation affects the conversion of indoleacetaldehyde to indoleacetic acid. The reasons for uncertainty are these: indoleacetaldehyde has not been proven to be an intermediary in auxin synthesis. It has not been isolated from the plant and has only once been synthesized and obtained pure. Gordon has not, I think, been able to use indoleacetaldehyde in any of his work. Assay of indoleacetaldehyde is achieved by conversion to auxin either by infusions of soil or by enzymes from leaves or by the aldehyde oxidase of milk, followed by biological assay of auxin formed. Since conversion is not complete, the assay involves complicated calculations.

Brown, Henbest and Jones (1952) who synthesized indoleacetaldehyde, tested the pure substance as an auxin or auxin precursor in various biological tests. They found it to be not more than 10 per cent as active as indoleacetic acid. Weber and Gordon (1952*b*) have used neutral ether extracts of cabbage as a crude source of indoleacetaldehyde in testing the conversion of the aldehyde to auxin. But Jones and coworkers (1952) have isolated indoleacetonitrile from neutral

extracts of cabbage and find that both the naturally occurring and synthetic substances are as active as auxin itself in some of the growth assay procedures, though not in others. On the other hand Gordon and Nieva (1949*a* and *b*) in two full papers have produced evidence that pineapple leaves, if extracted with ether, yield an aldehyde in the neutral fraction and this can be partly purified by reaction with dimedon or bisulphite. The aldehyde, when regenerated from its bisulphite compound, will react with soil enzymes or leaf enzymes to form auxin, but the conversion is not more than 10 per cent after 24 hours.

This subject, therefore, is in a state of flux. That radiation causes an immediate change in auxin formation seems certain but there still seem to be doubts as to what is the exact enzyme inhibited. Since this is such a fascinating piece of work one looks forward with particular interest to full publication.

What conclusions, if any, may be drawn from this partial survey? Two things stand out in my mind: first, that we cannot yet say in a single case that radiation directly or indirectly damages an enzyme; second, that this present position by no means rules out the possibility that enzymic damage is important. The study of the effect of radiation on enzymes is still in its infancy in spite of all the work that has been done.

Gray has pointed out that the amount of energy from radiation that is capable of preventing cell division is very small indeed and that damage must either be to a key enzyme, or be of a self-multiplying nature—or a break in continuity of an important structure.

Inactivation of a key enzyme might be the cause of the failure of DNA synthesis; break in continuity of a structure could be break up of DNA itself, or, for example, change in structure of mitochondria. The self-duplicating form of damage can be pictured as failure of enzyme synthesis.

Unfortunately we do not know what is the lifespan of cellular enzymes or at what stage new synthesis takes place.

Perhaps enzymes are stable once formed, or perhaps they wear out in use. Miller (1950) finds that depletion of protein in the diet leads to loss of liver enzymes and replacement of protein leads to their rapid restoration. Enzyme synthesis is therefore possible in the non-dividing mammalian cell and presumably takes place. In a number of experiments the manifestation of radiation damage has depended on metabolic activity and this could be imagined to be related to wearing out and non-replacement of enzymes. In bacteria, adaptive enzyme formation is not affected by doses that prevent division but one wonders whether one can equate bacteria that are extremely radioresistant with mammalian cells that are radiosensitive. But at present we have no evidence for failure of enzyme synthesis or failure of a key enzyme after radiation of a mammalian cell.

REFERENCES

ALTMAN, K. I., RICHMOND, J. E., and SOLOMON, K. (1951). *Biochim. biophys. acta*, **7**, 460.

ANDERSON, R. S. (1954). *Brit. J. Radiol.*, **27**, 56.

ASHWELL, G., and HICKMAN, J. (1952). *Proc. Soc. exp. Biol., N.Y.*, **80**, 407.

BACQ, Z. M., and ALEXANDER, J. (1955). Fundamentals of Radiobiology, p. 275. London: Butterworth.

BASSHAM, J. A., BENSON, A. A., KAY, L. D., HARRIS, A. Z., WILSON, A. T., and CALVIN, M. (1954). *J. Amer. chem. Soc.*, **76**, 1769.

BIEGEL, A. C. (1955). *Arch. Ophthal., Chicago.*, **54**, 392.

BILLEN, D., and LICHSTEIN, H. C. (1952). *J. Bact.*, **63**, 533.

BILLEN, D., STAPLETON, G. E., and HOLLAENDER, A. (1953). *J. Bact.*, **65**, 131.

BONNICHSEN, R. K., and HEVESY, G. (1955). *Acta chem. scand.*, **9**, 509.

BRANDT, C. L., FOREMAN, P. J., and SWENSON, P. A. (1951). *Science*, **113**, 383.

BROWN, J. B., HENBEST, H. B., and JONES, E. R. H. (1952). *Nature, Lond.*, **169**, 335.

BROWNIE, A. C., and GRANT, J. K. (1956). *Biochem. J.*, **62**, 29.

CIBIS, P. A., NOELL, W. K., and EICHEL, B. (1955). *Arch. Ophthal., Chicago*, **53**, 651.

CONWAY, E. J., GEOGHEGAN, H., and MCCORMACK, J. I. (1955). *J. Physiol.*, **130**, 427.

CRABTREE, H. G. (1935). *Biochem. J.*, **29**, 2334.

CRABTREE, H. G. (1936). *Biochem. J.*, **30**, 1622.

CRABTREE, H. G., and GRAY, L. H. (1939). *Brit. J. Radiol.*, **12**, 38.

DUBOIS, K. P., COCHRAN, K. W., and DOULL, J. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 422.
DUBOIS, K. P., COTTER, G. J., and PETERSEN, D. F. (1955). *Radiation Res.*, **2**, 79.
DUBOIS, K. P., and PETERSEN, D. F. (1954). *Annu. Rev. nucl. Sci.*, **4**, 351.
EICHEL, H. J., and SPIRTES, M. A. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 412.
ERRERA, M. (1955). Radiobiology Symposium, p. 93; ed. Bacq and Alexander. London: Butterworth.
FISCHER, M. A., COULTER, E. P., and COSTELLO, M. J. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 266.
GORDON, S. A. (1956). Progress in Radiobiology, in press. London: Butterworth.
GORDON, S. A., and NIEVA, F. S. (1949*a*). *Arch. Biochem.*, **20**, 356.
GORDON, S. A., and NIEVA, F. S. (1949*b*). *Arch. Biochem.*, **20**, 367.
GORDON, S. A., and WEBER, R. P. (1955). *Plant Physiol.*, **30**, 200.
HAGEN, U. (1955). *Experientia*, **11**, 485.
HEVESY, G., and FORSSBERG, A. (1951). *Nature, Lond.*, **168**, 692.
HEYNINGEN, R. VAN, PIRIE, A., and BOAG, J. W. (1954). *Biochem. J.* **56**, 372.
HOCHMAN, A., BLOCH, L., and FRANKENTHAL, L. (1953). *Brit. J. Radiol.*, **26**, 599.
JONES, E. H. R., HENBEST, H. B., SMITH, G. F., and BENTLEY, J. A. (1952). *Nature, Lond.*, **169**, 485.
KLECZKOWSKI, A. (1954). *Biochem. J.*, **56**, 345.
KUNKEL, H. O., and PHILLIPS, P. H. (1952). *Arch. Biochem. Biophys.*, **37**, 366.
LEMAY, M. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 337.
LIPETZ, L. E. (1955*a*) *Brit. J. Ophthal.*, **39**, 577.
LIPETZ, L. E. (1955*b*). *Radiation Res.*, **2**, 306.
MACFARLAND, M. L., PETERS, M. V., BALLANTYNE, R. M., and MCHENRY, E. W. (1950). *Amer. J. Physiol.*, **163**, 394.
MCDONALD, M. R. (1954). *Brit. J. Radiol.*, **27**, 62.
MILLER, L. L. (1950). *J. biol. Chem.*, **186**, 253.
MOLE, R. H. (1953). *Quart. J. exp. Physiol.*, **38**, 69.
NIZET, A., LAMBERT, S., and BACQ, Z. M. (1954). *Arch. int. Physiol.*, **62**, 129.
NOELL, W. K. (1951). *J. cell. comp. Physiol.*, **37**, 283.
ORD, M. G., and STOCKEN, L. A. (1953). *Physiol. Rev.*, **33**, 356.
PATT, H. M. (1955). *Ann. N.Y. Acad. Sci.*, **59**, 649.
PETERSEN, D. F., FITCH, F. W., and DUBOIS, K. P. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 394.
POWELL, W. F., and POLLARD, E. (1955). *Radiation Res.*, **2**, 109.
RAUCH, F. W., and STENSTROM, K. W. (1952). *Gastroenterology*, **20**, 595.
RICHMOND, J. E., ALTMAN, K. I., and SOLOMON, K. (1951). *J. biol. Chem.*, **190**, 817.
ROSENFELD, G., UNGAR, F., DORFMAN, R. I., and PINCUS, G. (1955). *Endocrinology*, **56**, 24.

RYSER, H., AEBI, H., and ZUPPINGER, A. (1954). *Experientia*, **10**, 304.
SCHRIER, K., ALTMAN, K. I., and HEMPELMANN, L. H. (1954). *Proc. Soc. exp. Biol., N.Y.*, **87**, 61.
SKOOG, S. A. (1935). *J. cell. comp. Physiol.*, **7**, 227.
SMITH, F., BUDDINGTON, W. G., and GREENAN, M. M. (1952). *Proc. Soc. exp. Biol., N.Y.*, **81**, 140.
SPIEGELMAN, S., BARON, L. S., and QUASTLER, H. (1951). *Fed. Proc.*, **10**, 130.
TERNER, C., EGGLESTON, L. V., and KREBS, H. A. (1950). *Biochem. J.*, **47**, 139.
THOMSON, J. F., and MIKUTA, E. T. (1954). *Proc. Soc. exp. Biol., N.Y.*, **86**, 487.
THOMSON, J. F., TOURTELLOTTE, W. W., and CARTTAR, M. S. (1952). *Proc. Soc. exp. Biol., N.Y.*, **80**, 268.
UNGAR, F., ROSENFELD, G., DORFMAN, R. I., and PINCUS, G. (1955). *Endocrinology*, **56**, 30.
WEBER, R. P., and GORDON, S. A. (1951*a*). *Argonne National Laboratory Report* 4676, 79.
WEBER, R. P., and GORDON, S. A. (1951*b*). *Argonne National Laboratory Report* 4713, 91.
WEBER, R. P., and GORDON, S. A. (1951*c*). *Argonne National Laboratory Report* 4745, 81.
WEBER, R. P., and GORDON, S. A. (1952*a*). *Argonne National Laboratory Report* 4794, 113.
WEBER, R. P., and GORDON, S. A. (1952*b*). *Argonne National Laboratory Report* 4840, 71.
YANOFSKY, C. (1953). *J. Bact.*, **65**, 383.

DISCUSSION

Latarjet: You quoted experiments of Nizet, Lambert and Bacq, who found stimulation of haemin synthesis by irradiated reticulocytes. The stimulation of normal biological functions by radiations themselves or by their chemical intermediates has always been surprising, and has even been questioned. May I point out in connection with this that Dr. Monier in my laboratory has treated pepsin with very small amounts of an organic peroxide, and has found an increase, by a factor of 1·5, in the enzymatic activity.

Van Bekkum: Dr. Pirie, you quoted data from Altman, Richmond and Solomon in your Table II; as far as I know, these experiments have not been repeated by others so far and the evidence in support of an increase in fatty acid synthesis is very meagre indeed, because each figure you showed was derived from one rabbit only, and the authors did not indicate the variation in the control rabbits.

Pirie: It was whole body irradiation, so they could not take one leg and use the other as a control. Have you got similar reservations about the data on increase in synthesis of haemin and globin?

Van Bekkum: I do not have reservations of the same kind but I have

some reservations, because these data do not agree with what we know about haemoglobin synthesis *in vivo* from other studies, for instance those of Prof. de Hevesy.

Lajtha: In connection with haemoglobin synthesis, we have done some irradiation of bone marrow *in vitro* and *in vivo*. In our *in vitro* studies we irradiated with doses of up to 5,000 r and then studied the iron uptake with a high resolution autoradiography. We could detect no increase or decrease in the uptake, neither in normoblasts nor in erythrocytes. *In vivo* we gave up to 220 r. Bacq and Nizet gave 800 r *in vivo*, and they gave huge doses, up to 100,000 r, *in vitro*.

Alexander: They gave those large doses *in vitro* because small doses had no effect; they irradiated reticulocytes outside the dog and they got no decisive effect until they reached 100,000 r.

Gray: They did get one effect at about 500 r. One gets the impression that with the four dogs the results were rather variable. In one case an effect was obtained at 500 r and in another none was observed at 10,000 r.

Alexander: I know the details of this work fairly well. The conclusion which is based on limited data only is that plasma from an irradiated dog was sufficient to stimulate unirradiated reticulocytes to greater haemoglobin synthesis. The results about which I think there can be no doubt are that the reticulocytes taken from dogs irradiated with 500 r synthesize haemin at a considerably faster rate than those which had not been irradiated.

Lajtha: How long after irradiation did they take blood from the dog to measure the reticulocyte stimulation?

Alexander: They took it as soon as they could.

With regard to the general problem of changes in enzyme activity after irradiation of animals, Prof. Bacq and I reached the conclusion a few years ago on reviewing the literature that there seem to be no immediate decreases and in a few cases there was an increase in activity. The effect of radiation may be to disturb the internal barriers of the cell so as to allow enzymes to get access to sites from which they are normally excluded (Bacq, Z. M., and Alexander, J. (1955). Fundamentals of Radiobiology, p. 187). In this way one would find an increase in activity if one looks immediately after irradiation. But after a time there will be a loss in activity due to the mutual destruction of enzymes which radiation had allowed to come together. An experiment is under way at the moment to test this hypothesis. Errera found that the rigidity of a nucleoprotein gel obtained by placing nuclei in water was decreased by irradiation. The effect was greater when the intact cells were irradiated than when the isolated nucleoprotein was irradiated. With Prof. Bacq we have now done some experiments on spleen nuclei; if one puts spleen nuclei into water they swell very much but do not go into true solution, since on high speed centrifuging all the u.v.-absorbing material (DNA-protein) goes to the bottom of the cell. On standing for as little as 30 minutes at room temperature, but not at 0° C, this is no longer the case and the u.v.-absorbing material is not spun down. It looks as if an enzyme is liberated during the swelling of the nuclei in water, which attacks the nucleoprotein gel and changes it from a gel

to a sol. If the intact cells are irradiated and the nuclei isolated subsequently, then the gel obtained is partially damaged. Irradiation of the isolated nuclei with small doses does not have this effect on the nucleoprotein gel. This may be interpreted as the release, on irradiation of the cell, of an enzyme which is able to break down this nucleoprotein gel, turning it into a sol.

Butler: The question which Dr. Pirie has been discussing is whether in addition to effects on preformed enzymes there are effects on the synthesis of enzymes, and one step in the synthetic process is, or may be, the incorporation of amino acid. We have done a few experiments in which we looked at the effects of irradiation on the incorporation of amino acid in the proteins, actually of the rat liver. I know that other experiments of that type have been done with rather inconsistent results. It might be of interest to you in connection with the stimulations you mentioned that we do find a stimulation of incorporation by rather small doses of X-rays. It is not known what effect this has on synthesis of proteins, but the radiation certainly has some effect on the incorporation reaction.

Pirie: I think that the question of whether enzyme synthesis is upset or not is one of the most interesting ones, and I wonder whether the fact that in bacteria and yeast it is not affected is really relevant for the very much more radiosensitive mammalian cell. It is difficult to get data about enzyme lifespans in mammalian cells, but there certainly are situations where enzymes are synthesized very rapidly. Miller found that if he starved a rat, i.e. gave it a low protein diet, then there were quite rapid changes in enzymes of the liver, and on replacing protein in food the enzymes returned to normal activity in a few days. That is a situation where enzyme synthesis is going on and could be studied.

Alper: Since the question of relative sensitivity of bacteria and mammalian cells comes up quite often, I would like to point out here that the sensitivity is not as different as is generally supposed. The sort of doses which give, for instance, chromosome breaks in cells, are not very far off the sort of doses with which you get long forms and much increased lag. The doses which give killing effects in bacteria are not really so different from those which kill mammalian cells. It is often said that you cannot compare them, but I think this is not correct.

EFFECTS OF X-RAYS AND RADIOMIMETIC AGENTS ON NUCLEIC ACIDS AND NUCLEOPROTEINS

J. A. V. Butler

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London

I INTERPRET my function as being to give an account of present views on nucleic acid and nucleoprotein structure and the chemical effects of irradiation, as a background for the more specialized discussions which will follow on the actual effect of ionizing radiations on metabolic processes in which these substances are known or suspected to take part. The discussion of DNA must begin with the structure proposed by Crick and Watson (1953) which, although it may be subject to modification in some minor respects, has proved adequate so far to accommodate the known facts. In this structure, as is well known, two complementary nucleotide chains are held together by hydrogen bonds between the bases guanine and cytosine and adenine and thymine. Numerous measurements of molecular weight have given values of the order $6–8 \times 10^6$. This implies a chain length of approximately 5×10^4 Å or 5×10^{-4} cm. As determined by physical measurements, the actual length of the particle is considerably less than this, viz. $4–6 \times 10^3$ Å (Sadron, 1955). It follows that the particle, although a fairly rigid structure, must be bent or coiled. When studied by Shooter and Butler (1955) in the ultracentrifuge at low concentrations, a very considerable range of sedimentation constants was observed (often from $S = 10$ to $S = 40$), so that there must be present a variety of fixed shapes or sizes. Since one source (calf thymus) has given a variety of products, we have to conclude that the product is sensitive to the mode of preparation (*e.g.* by enzyme actions).

It must also be realized that the structure of DNA is not a

very stable one. It is disrupted by heat in water or salt solutions, and is also sensitive to the action of dilute acids or alkalies. Heating (e.g. at 100° for 15 minutes in water) causes a great decrease in viscosity of solutions of DNA, with no very marked change of sedimentation behaviour (Zamenhof, Alexander and Leidy, 1954; Doty and Rice, 1955). There have been conflicting interpretations of this, due mainly to differences of conditions. However, there is no doubt that a considerable amount of disruption of the hydrogen-bonded structure occurs on heating, with a decrease in molecular weight which depends partly on the specimen used and partly on the concentration. On the basis of their results Dekker and Schachman (1954) have suggested that the nucleotide thread is interrupted at various points and that the DNA particle is held together by hydrogen bonds between the overlapping segments. It is, of course, difficult to establish whether such interruptions are (1) originally present in the DNA; (2) produced during the preparation by the DNAse present in the cells; (3) not originally present, but only caused by heat. The drop in molecular weight (1/4—1/6) on heating was found by Shooter, Pain and Butler (1956) to be much less in good specimens than that found by Dekker and Schachman. It has also been shown by Shooter and Butler (1956) that degradation occurs at quite a rapid rate in the cell homogenates and even in isolated (aqueous) nucleoprotein. The third possibility would imply "weak points" in the nucleotide at which dissociation by heat occurs, which might be the case if a few of the PO_4 bonds are triply esterified and thus easily hydrolysed. No independent evidence of this has been obtained. However, comparable heating in the solid state produces no degradation, so that the effect of heat may involve hydrolytic changes.

The effect of ionizing radiations on DNA has been studied under a variety of circumstances. In aqueous solution (0·1 per cent) the characteristic high viscosity of DNA is greatly reduced by comparatively small doses of radiation (see Fig.1), about 8000 r being required to reduce the intrinsic viscosity by one half (at 0·1 per cent). This is due mainly to a

decrease in particle size, but the relation between it and molecular weight changes is not a simple one.

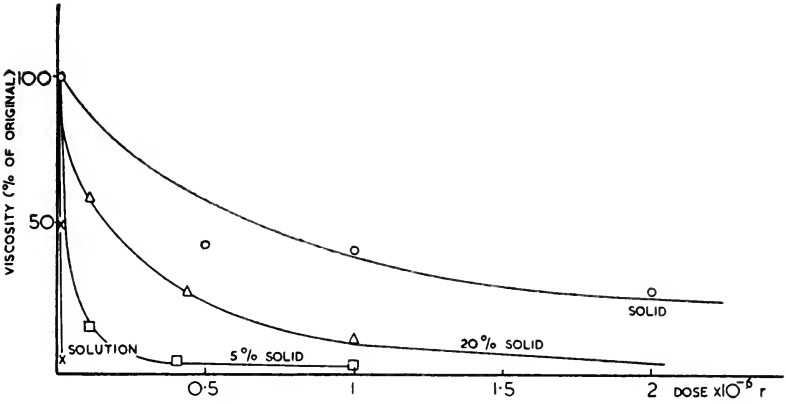


FIG. 1. Effect of 15 MEV electrons on solid and aqueous DNA. (Measurements by R. H. Pain on materials irradiated by Prof. J. Rotblat.)

Chemical changes can also be observed in aqueous solutions although large doses of radiation are required to produce easily measured effects. Among the reactions which have

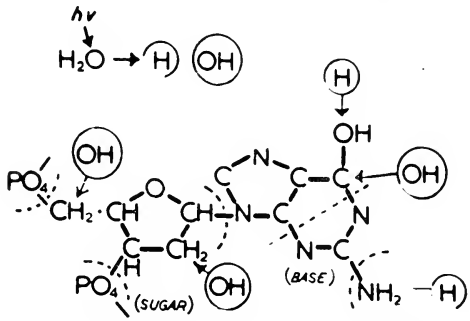


FIG. 2. Some chemical effects of radicals on DNA in aqueous solution.

been observed (Fig. 2) are (1) deamination of the bases; (2) dehydroxylation; (3) fission of the sugar-base linkage and in some cases breakage of the pyrimidine ring; (4) oxidation of the sugar moiety and (5) breakage of the nucleotide chains

and liberation of inorganic phosphate; (5) occurs normally as a consequence of (4) (Scholes and Weiss, 1952; Butler and Conway, 1953).

All these chemical effects are primarily radical reactions and can be produced by —OH radicals formed in other ways. The reactions of —H are not so well defined, except in so

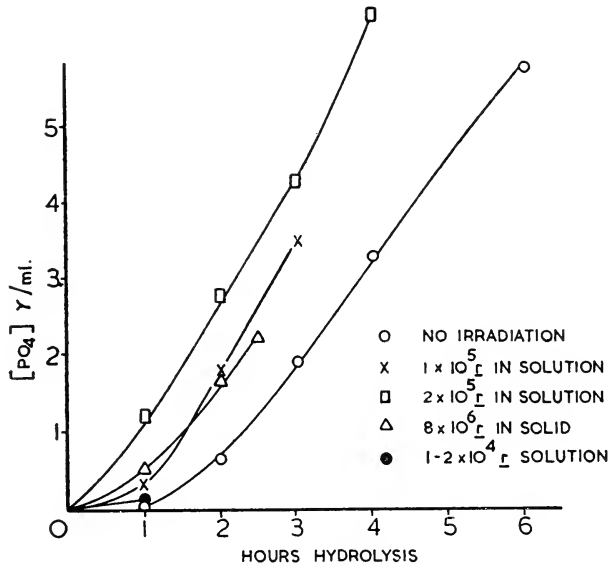


Fig. 3. Hydrolysis of DNA by heating with sulphuric acid after irradiation (Butler and Simson, 1954).

far as it combines with oxygen to form the oxidizing radical O_2H. The liberation of inorganic phosphate varies with the square of the dose, as is to be expected since *two* phosphate ester bonds have to be broken in order to liberate PO_4 (Butler and Conway, 1953). It is not easy to demonstrate directly the breakage of the nucleotide chains. The effect of single breakage is to liberate a terminal phosphate group, but it can be shown that after irradiation free phosphate is more quickly liberated on acid hydrolysis (Butler and Simson, 1954; see also Fig. 3).

It had been found that the dose required (in solution), expressed in r units, to bring about a given amount of change increases directly with the concentration (Cox *et al.*, 1955). This is, of course, a characteristic of indirect action and will be true if a constant fraction of the radicals is effective in bringing about the observed change.

In dilute solutions about 100 ionizations are required per molecule in the whole solution to reduce the viscosity by one half, i.e. the process of degradation is comparatively inefficient.

When irradiated in the *solid* state, much greater doses are required to bring about a given change, about 5×10^5 r is required to reduce the intrinsic viscosity by one half, i.e. the factor for solid/dilute solution (0·1 per cent) is of the order of 100 for viscosity (Fig. 1). However, the *actual* efficiency per ionization in producing a viscosity change is indeed greater in the solid than in the solution, since as pointed out by Alexander and Stacey (1955) less than 3 ionizations per molecule produced in the solid do enough damage to reduce the molecular weight by one half, and Fluke, Drew and Pollard (1952) found that about one ionization will inactivate *Pneumococcus*-transforming principle. The greater sensitivity per ionization produced in the solid state might be anticipated, as in dilute solution many of the ionizations give rise to radicals at considerable distances from the DNA particles, and will recombine with each other before reaching them.

This does not mean that the effect of radicals *in vivo* is necessarily insignificant. The overall effect of irradiation is greater in solution because ionizations occurring over a considerable volume are effective by the radical mechanism. When only a comparatively small amount of water is present, the effect of ionizations in the water is relatively greater than in dilute solutions and of the same order as that in an equal volume of DNA; e.g. in 20 per cent DNA the sensitivity to a given dose of radiation is 4 or 5 times that in solid DNA.

The effect of X-rays is also similar to that of heat in causing a denaturation of the DNA. This is primarily a breakage of hydrogen bonding between the threads. The result is shown

up in the fact that heat denaturation occurs more easily after irradiation (Fig. 4). This effect occurs both in solution and in the solid state, since it has also been found by Cox and co-workers (1955), by titration curves, that X-ray treatment

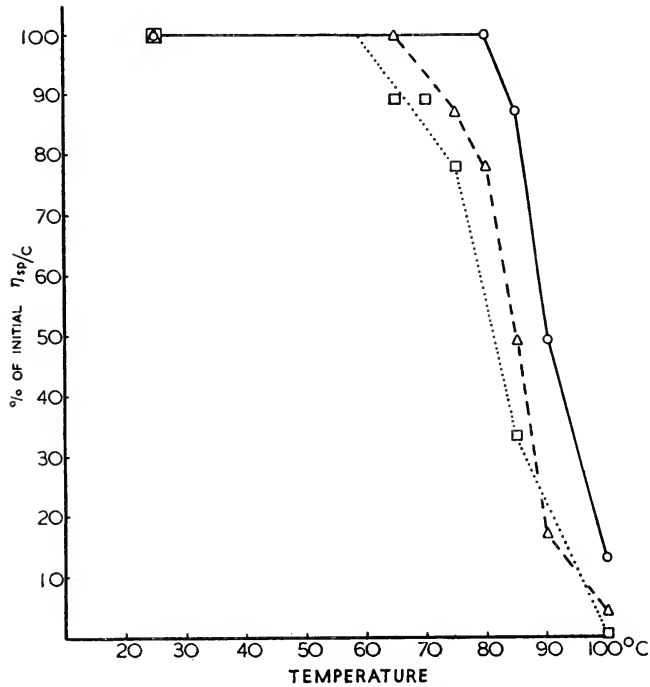


Fig. 4. Effect of irradiation on the temperature required to denature DNA in 0·01 per cent aqueous solution.
——0—— Unirradiated DNA.
– – △ – – Irradiated with 10^6 r of 15 Mev electrons.
. . . . □ . . . . Irradiated with 4×10^6 r of 15 Mev electrons.
(Measurements by R. H. Pain on materials irradiated by Prof. Rotblat.)

of solutions with doses of 8000 r causes a considerable breakage of the hydrogen bonding. It is clear that this is a fairly efficient process.

When we ask what biological effects are produced by these changes we are in a much more difficult position. If DNA is

the genic substance it is probable that chemical damage of any kind will have some effect, but it is difficult to particularize as we do not know how the genetic information is carried. If reduplication occurs by each base attracting its complement as in the Crick and Watson scheme, it is clear that loss of $—NH_2$ or —OH groups by bases will prevent their reduplication at this particular point. A break in a nucleotide chain might make it impossible for the new particle of DNA to be formed intact. However, it is not at present certain that DNA duplication occurs in this way and there are other possibilities in which some damage of the molecules might be possible without impairing the genetic character. It is noteworthy that Stent (1955) found that a considerable amount of breakage of the nucleotide chains of phage could occur without any loss of activity.

It is difficult to see how the mere breakage of hydrogen bonds between the nucleotide chains could cause permanent genic damage as the reduplication, at least on the Crick and Watson scheme, involves the separation of the two fibres, unless the particle can only take part in the duplicating process if it is intact and complete. It would be expected that a small degree of hydrogen bond breakage could easily be made good. This might possibly happen in some "reactivation" processes. However, attempts to reactivate trypsin which had been inactivated to the extent of 50 per cent by irradiation in the solid state by exposure to ultraviolet, infrared and heating at 100° were unsuccessful (Butler and Phillips, 1956, unpublished experiments). It might, however, be possible to reverse slight degrees of damage.

It must also be remembered that the DNA is actually present in somatic cells combined with histone. The histone is known to be complex and to contain several substances of different composition. However, since the *total* amount of the bases present in the histone corresponds to at least 85 per cent with the total amount of phosphate (Davison and Butler, 1956), it is reasonable to suppose that all or nearly all the bases are attached to phosphate groups of DNA. We do not

know what the function of this part of the nucleoprotein system is.

The influence of this protein on radiation effects is not easily assessed. It might be expected *a priori* that histone would have a protective effect on DNA by mopping up radicals, which would be prevented from reaching the DNA. No accurate experiments on this have been made as it is difficult to be sure that DNA has been recovered quantitatively from the nucleoprotein, and slow degradative changes occur in the nucleoprotein on standing.

It may be useful to estimate the degree of damage in the chromosome particle by different processes. If we take the molecular weight of DNA as 6×10^6, it can be estimated that 10^6 r will cause about 7 ionizations within each molecule, and we have seen that 2–3 ionizations will cause a considerable amount of damage, which can be expressed as sufficient to reduce the viscosity in aqueous solution by half. In the hydrated state in the presence of water it may be expected that this will be increased by several times at least, i.e. about 10^5 r will cause the same degree of damage (expressed as $0{\cdot}5$ of the original viscosity). As there are 10^5 particles in the chromosome, this means that the probability of any one DNA particle in the chromosome being damaged to this extent by exposure to 1 r is of the order of 10^{-5}.

It is estimated that (in mice) the probability of mutation in any one locus is between 3 and 200×10^{-8} per r unit (Russell, 1952). If we take 10^{-7} or 10^{-6} as possible values, we see that the probability of producing a specific mutation is about 10^{-1} or 10^{-2} of that of damaging, to the extent mentioned, the DNA particle which carried the gene in question. This means that a considerable amount of damage can be done to a DNA particle without affecting a specific gene carried by it. This can be expressed in another way by saying that the locus in question is not greater than the order of 10^{-1} or 10^{-2} of the size of an average DNA particle. There are, of course, great uncertainties in many of the quantities entering into this calculation.

Let us turn now to the so-called radiomimetic agents like the nitrogen mustards, which are capable of producing (1) chromosome breaks, and (2) mutations. *In vitro* they combine with DNA in various ways, especially with $—NH_2$ and —OH. Combination with phosphate groups may also occur but will in general be rather labile. The effect of combination with the $—NH_2$ and —OH groups is to break up the hydrogen-bonded structure of DNA, which has been shown to be followed by a slow degenerative breakage of nucleotide chains, probably caused by hydrolysis of triply esterified phosphate (see Butler, Gilbert and James, 1952). It might be asked if this kind of reaction actually occurs *in vivo*, especially since there are so many competing molecules such as proteins with which reaction could occur.

It has been found that (1) when a nitrogen mustard (in this case a radioactively labelled phenylalanine nitrogen mustard) reacts with intact deoxyribonucleoprotein in aqueous medium, reaction occurs with both the DNA part and the histone part in the ratio of about 2 : 1 (Table I). This means that nitrogen

Table I

REACTION OF A NITROGEN MUSTARD *p*-DI(2-CHLOROETHYL)AMINO-DL-^{14}C-PHENYLALANINE WITH DEOXYRIBONUCLEOPROTEIN EXTRACTED FROM VARIOUS TISSUES.

(Experiments by J. A. V. Butler and A. R. Crathorn.)

	Activity in DNA Fraction	*Activity in Protein*
Calf Thymus (dried preparation)	3·24	1·03
Rat Thymus (fresh wet preparation)	4·27	2·09
Spleen preparation	2·62	2·20

mustards can easily react with DNA even when the latter are combined with histone; (2) when the same nitrogen mustard is introduced into the rat, within a period of 1–3 hours (and possibly shorter times) reaction has occurred with DNA and RNA in all the cell fractions and in all the organs examined (Table II). These substances therefore react very extensively with nucleic acids *in vivo*.

Table II

SPECIFIC RADIOACTIVITIES OF PROTEIN AND NUCLEIC ACID FRACTIONS FROM RATS ADMINISTERED WITH A ^{14}C-LABELLED NITROGEN MUSTARD *p*-DI(2-CHLOROETHYL)AMINO-DL-PHENYLALANINE. (EXPERIMENTS BY P. COHN.)

Tissue	*Dose*	*Protein*	*RNA**	*DNA**
Spleen	10 mg./kg.: rats killed after one or two days.	0·017	0·004	0·010
Thymus		0·021	0·007	0·010
Liver (mitochondria)	10 mg./kg. twice at intervals of 2 days: killed on 4th day.	0·083	0·037	—
Liver (microsomes)		0·073	0·037	—
Kidney (supernatant)		0·33	0·20	—

* Purified by precipitation with ethanol.

In the absence of precise knowledge of the functions of these substances, it is difficult to say what effects might be expected to follow from these reactions. However, it is obvious that reaction with DNA and RNA will not only interfere with their ability to reproduce themselves but also with their metabolic functions.

Table III

COMPARISON OF INHIBITORY EFFECT OF SOME AROMATIC NITROGEN MUSTARDS ON THE "EXCHANGE REACTION" IN *Staphylococcus aureus* WITH THE INHIBITION OF GROWTH OF THE TRANSPLANTED WALKER CARCINOMA.

(Experiments by G. D. Hunter and A. R. Crathorn.)

Formula $R = (Cl \cdot CH_2CH_2)_2N \cdot C_6H_4$	*Inhibition of growth of Walker carcinoma*	*Percentage inhibition of exchange reaction with phenylalanine*
$R \cdot CH_2CO_2H$	+++	95
$R \cdot (CH_2)_3CO_2H$	++++	97
$R(CH_2)_4CO_2H$	—	5
$RO(CH_2)_3 \cdot CO_2H$	+	44
$RO(CH_2)_4CO_2H$	++	49
DL-$R \cdot CH_2CH(NH_2) \cdot CO_2H$	+++	46
D-$R \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$	+	40
L-$R \cdot CH_2CH(NH_2)CO_2H$	++++	78

It has been found by my colleagues Dr. Hunter and Dr. Crathorn, that with *Staphylococcus aureus* the effect of a series of nitrogen mustards in inhibiting the incorporation of an amino acid under conditions of starvation runs parallel to their effects on tumour inhibition (Table III, Hunter and Crathorn, 1956, unpublished experiments). There are thus reasonable grounds for expecting that the reaction of the nitrogen mustards with nucleic acids in the cell will have metabolic consequences and will also affect the genic characteristics.

Acknowledgement

I am indebted to my colleagues Drs. A. R. Crathorn, P. Cohn, G. D. Hunter and D. M. Phillips for permitting me to quote unpublished results.

REFERENCES

ALEXANDER, P., and STACEY, K. A. (1955). IV Int. Conf. Radiobiol., Edinburgh : Oliver & Boyd.

BUTLER, J. A. V., and CONWAY, B. E. (1953). *Proc. Roy. Soc.*, (B), **161**, 562.

BUTLER, J. A. V., GILBERT, L., and JAMES, D. W. F. (1952). *J. chem. Soc.*, 3268.

BUTLER, J. A. V., and SIMSON, P. (1954). Liège Radiobiology Symposium, p. 46.

COX, R. A., OVEREND, W. G., PEACOCKE, A. R., and NELSON, S. (1955). *Nature, Lond.*, **176**, 919.

CRICK, F. A. C., and WATSON, J. D. (1953). *Nature, Lond.*, **171**, 737.

DAVISON, P. F., and BUTLER, J. A. V. (1956). *Biochim. biophys. acta*, in press.

DEKKER, C. A., and SCHACHMAN, H. K. (1954). *Proc. nat. Acad. Sci., Wash.*, **40**, 894.

DOTY, P., and RICE, S. A. (1955). *Biochim. biophys. acta*, **16**, 446.

FLUKE, D., DREW, R., and POLLARD, E. (1952). *Proc. nat. Acad. Sci., Wash.*, **38**, 180.

RUSSELL, W. L. (1952). *Cold Spr. Harb. Symp. quant. Biol.*, **16**, 327.

SADRON, C. (1955). III Int. Congr. Biochem., p. 125.

SCHOLES, G., and WEISS, J. (1952). *Exp. Cell Res.*, Suppl. **2**, 219.

SHOOTER, K. V., and BUTLER, J. A. V. (1955). *Nature, Lond*, **175**, 500.

SHOOTER, K. V., and BUTLER, J. A. V. (1956). *Nature, Lond.*, **177**, 1033.

SHOOTER, K. V., PAIN, R. H., and BUTLER, J. A. V. (1956). *Biochim. biophys. acta*, in press.

STENT, G. (1955). *J. gen. Physiol.*, **38**, 853.

ZAMENHOF, S., ALEXANDER, H., and LEIDY, G. (1954). *J. exp. Med.*, **98**, 373.

DISCUSSION

Koller: Why do you think that mutation may involve damage to the gene molecule? Why not assume that mutation is caused by changing the sequence of purine-pyrimidine bases in the DNA?

Butler: You would have to break it up in order to change the sequence. There is no easy chemical way of changing the sequence.

Haddow: You were thinking of cytology?

Koller: Yes. Owing to the fact that mutation can be reversed and the original gene structure reformed, the term "damage", which is commonly used by chemists and physicists, should be more clearly specified.

With regard to the question of hydrogen bonding, can we assume that reduplication of the chromosome would involve the breaking of all the hydrogen bonds in the DNA, which is a very high number? Are there other possibilities to explain chromosome duplication and separation?

Spiegelman: I think that the doubled molecule has really been eliminated by the Levinthal experiment which shows that you don't get randomization of the ^{32}P making duplicates.

Butler: I heard that Mazia has the opposite results.

Spiegelman: I don't think the two types of experiments can be compared. Mazia studied chromosomal multiplication and Levinthal the duplication of viral DNA. Levinthal's results are consistent with the obvious duplication mechanism deducible from the Watson-Crick structure. He starts out with virus particles heavily labelled with ^{32}P and examines the distribution of ^{32}P in the progeny particles produced. An electron-sensitive photographic emulsion is used for the measurement of the radioactivity of a single virus particle or a single DNA molecule. He finds that 40 per cent of the DNA is contained in one large piece which replicates to produce two particles, each containing half of the original atoms. No further distribution of the original atoms takes place with subsequent replication.

Alper: This whole experiment seems a bit mysterious if it is done with phage, when it is considered to be fairly well established that the first thing that happens when a phage gets inside a bacterium is that it just breaks up completely.

Spiegelman: No, all that happens is that DNA goes in and leaves the protein of the phage behind.

Alper: But it is not recognizable as phage chromosome for quite a while, so that it is hard to see how things should come together, the hot with the hot and the cold with the cold, so to speak.

Spiegelman: I don't understand why you believe that it falls apart completely.

Alper: It depends on what one means by completely. You cannot pick up phage recognizable as phage, or indeed any virus as virus, for quite a long period.

Spiegelman: One cannot find infectious virus particles until the coats and tails have been synthesized and put together, since these are necessary for attachment and infection. However, the virus DNA is immediately recognizable in such instances, e.g., T2 which contains 5-OH-methylcytosine.

Alper: Yes, the phage DNA, but I am talking about the actual biological continuity of the phage particle as such. Now you are talking about these threads presumably as carriers of the genetic material. What is supposed to happen when phage gets into a bacterium is that the genetic components come apart and they are somehow reconstituted.

Spiegelman: I don't think that is true.

Alper: I think it is.

Mitchell: I would like to make a suggestion about the therapeutic action of radiation (see Mitchell, J. S. (1956), *J. Colloid Sci.*, in press). It is well known that a dose of say 2,250 r of gamma-rays produces permanent healing of a typical small carcinoma of the skin in man. The DNA content per nucleus in such a tumour was found by u.v. photomicrographic absorption methods to be $7 \cdot 8 \times 10^{-12}$ g. The mean diameter of the tumour cell nuclei was 6·86 microns. The number of ion pairs within the material of the nuclei thus corresponds to a number of DNA molecules of arithmetic mean molecular weight almost exactly 7 millions. This may suggest inhibition of reduplication, but that hypothesis is not essential to the argument. While classical target theory is obviously not the mechanism involved, one must think of a macromolecular lesion of DNA or DNA-protein as the basis for the therapeutic effect.

A possible experimental test is that one factor in radiosensitivity would be the molecular weight of the DNA within the cell. I have already started by methyl-green staining of sections cut in the same block from radio-curable and radio-incurable tumours of the uterine cervix. In the first pair of specimens there was very much less intense methyl-green staining in the radio-incurable case. Much further work is required.

Lajtha: I should like to ask Prof. Butler three questions, the first one being whether he thinks that DNA in the cell may be more radioresistant than DNA in solution. We labelled bone marrow cells *in vitro* with ^{14}C-adenine and then irradiated with 500 r and followed whether up to 48 hours there was any loss of labelled DNA; we found no loss after 500 r. We have repeated the experiment using 5,000 r, not with labelling but just following the staining reaction of these cells with methyl green-pyronin and Feulgen, and were unable to detect any significant decrease in stainability. I don't think our technique is very sensitive, so there may have been some small losses not detectable, but certainly no significant loss.

Secondly, how can one explain the differential radiosensitivity of the incorporation of ^{14}C-formate into DNA on the hydrogen bond breakage theory? Thirdly, what does Prof. Butler think is the mechanism of the indirect radiation effects on bone marrow when the spleen is irradiated?

Butler: As to the first question, that is really a type of chemical experiment and you would picture it as similar to a test-tube experiment in which one observed the liberation of adenine.

Lajtha: Yes.

Butler: I think it would require more than 5,000 r to produce any observable effect.

Lajtha: We get about $12\frac{1}{2}$ million ^{14}C atoms incorporated into DNA!

Butler: You would require a fairly heavy dose in order to produce detectable liberation of adenine. I think even in solution you require fairly heavy doses, about 100,000 r; 8,000 r will produce perhaps one break in nucleotide chains, a very small fraction of the whole. With regard to the other questions, these involve synthetic reactions and I don't know what the synthetic reactions are in the mechanism of synthesis of DNA. It is true that radiation has a marked effect on spleen and thymus, and it has been reported that the DNA obtained from the radiated spleen and thymus is relatively broken down. We tried to repeat that, but we were not able to detect any difference in the isolated DNA. What happens, of course, is that the radiation kills the cells and you may get DNA which has been metabolically damaged by enzymes from dying cells.

Forssberg: In vivo irradiation sometimes causes a marked degradation. Bachmann and Harbers irradiated Walker carcinoma with 5,000 r and isolated two DNA fractions according to Bendich, DNA_I having a high and DNA_{II} a lower molecular weight. In the non-irradiated material there was about three times as much high molecular DNA as low molecular; but after irradiation with 5,000 r, within two hours the ratio was about 0·2–0·3; so there is a very high grade depolymerization in this carcinoma. Also the incorporation rates of isotopes are different in these two fractions.

Gray: I would like to ask Prof. Butler for his observations on the paper by Dr. Kaufmann and his group which seemed to me very interesting as stressing the great sensitivity of the nucleoprotein as distinct from DNA. They performed several experiments. In the first series of experiments they formed a gel from calf thymus, irradiated the gel with 1,000 r and then studied the fall in viscosity. This was much more rapid than when they had high salt concentration present which dissociated the nucleoprotein. In another experiment the effect of radiation on a dilute solution of an artificial nucleoprotein obtained by mixing calf thymus DNA with bovine albumin was compared with the effects of radiation on dilute solutions of the DNA and the protein separately. After exposure to 1,000 r the fall in viscosity was very much greater in the former case, that is, when the nucleoprotein was irradiated. It is of interest that the DNA used in these experiments was derived from calf thymocytes and I wonder whether these experiments provide a clue to the rather remarkable radiosensitivity of these cells.

Butler: This is rather reminiscent of Anderson's experiment at Oak Ridge. The only thing that one can say about it is that you have there a complex, a gel-like system of filaments which are bound together in some way, and it certainly is true that very small doses of radiation break down this structure. However, it is a very labile binding, because it is affected even by shearing forces. What the mechanism of it is I don't know; Dr. Hollaender may.

Hollaender: This nucleoprotein, which Anderson has prepared from thymus, spleen, and other organs, responds readily to as little as 25 roentgens and is almost like water (Fig. 1). It behaves in many ways

like polymer systems which have been studied previously. It can be protected against irradiation by certain protective substances which will be discussed later, and it can be studied quantitatively. In many ways the preparation resembles the chromosome in the cell, at least as we picture it from microdissection studies. It has, for example, pronounced elasticity. It will break down in response to shearing stress, as Prof. Butler pointed out, but the breakdown need not interfere with the observation of radiation effect. Mr. Fisher, who is now working in our laboratory, has devised a viscometer in which the viscosity may be measured without previously subjecting the preparation to a shearing

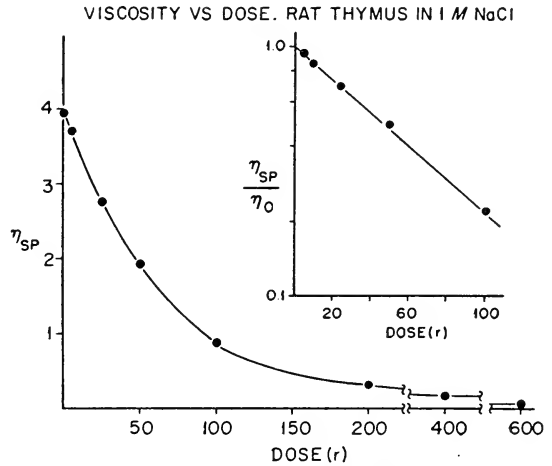


FIG. 1. (Hollaender). Effect of X-rays on viscosity of crude rat thymus preparation (W. Fisher, unpublished).

field. With it he has been able to get very reproducible results. It should be pointed out that sodium deoxyribonucleate is also sensitive to mechanical stresses, and can be broken down during isolation, but it is much more resistant than the nucleoprotein.

I would like to say that the nucleoprotein which Kaufmann has isolated is in many ways much closer to what exists in the living cell than the pure salt suspension which has been isolated. Unfortunately these nucleoproteins cannot be dried, but it might be possible to reduce them to a very low temperature where a direct effect could be observed.

Alper: What about the oxygen effect?

Hollaender: These are very viscous and the moment you bubble oxygen through you break down the pattern in which they are organized. We have not learned how oxygen may be removed and so far it cannot be removed; the moment you bubble nitrogen or any other gas

through it or put a vacuum on so that you get the gas out, you break the structure down.

Gray: If you remove the oxygen chemically then they are protected?

Hollaender: Yes. In the curve shown in Fig. 1 the change in viscosity with increasing irradiation was seen. Now if AET is added (Fig. 2), no effect on viscosity is observed without irradiation (upper curve). With 250 r a very large decrease is observed without the AET (middle curve), but with small concentrations a large protective effect is seen. With 900 r (lower curve) more AET is needed for maximal protection. Much more complete protection is observed with lower doses of radiation.

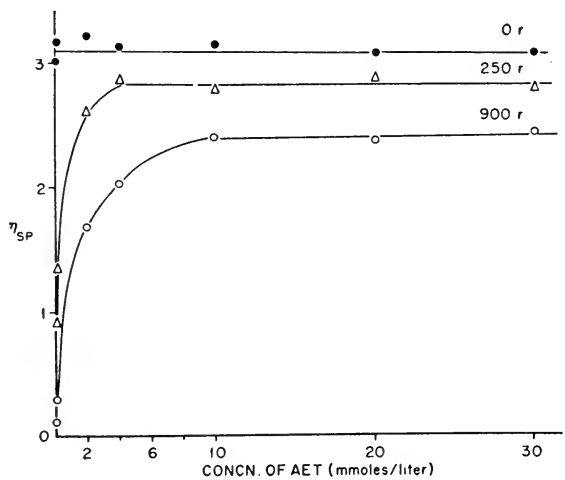


FIG. 2. (Hollaender). Protective effect of *S*-β-aminoethyl*iso*thiouronium bromide HBr on crude rat thymus preparation (W. Fisher, unpublished).

There is, however, a viscosity change which AET will not protect against.

de Hevesy: Dr. Lajtha mentioned the effect of the irradiation of the spleen. Is this effect not due to an interference with the formation of a humoral substance and is the much greater effectiveness of the whole-body radiation not partly due to the supression of the formation of this substance in the whole organism?

Alexander: With regard to the irradiation of DNA *in vitro*, the resistance of DNA to radiation, which has been stressed by Butler, is more apparent than real. Dr. K. A. Stacey (1955, *Int. Conf. Radiobiol.*, Cambridge, 1954, B.E.C.C. report, **32**, 29) at our Institute irradiated DNA from herring sperm in the dry state and then measured its molecular weight by light scattering. From the change in molecular weight the energy needed to break one polynucleotide bond can be determined.

If the measurements are made in dilute salt solution, quite high doses are required to decrease the apparent molecular weight. When unirradiated herring sperm DNA is dissolved in concentrated urea solution, its molecular weight is halved, and is now only 3 million instead of 6 million (Alexander and Stacey, (1955), *Biochem. J.*, **60**, 194). If the irradiated DNA is measured in solution containing urea then relatively small doses produce a decrease in molecular weight. A typical experiment is as follows: a sample of DNA containing about 5 per cent of moisture is irradiated with 2×10^5 rad and its molecular weight measured dissolved

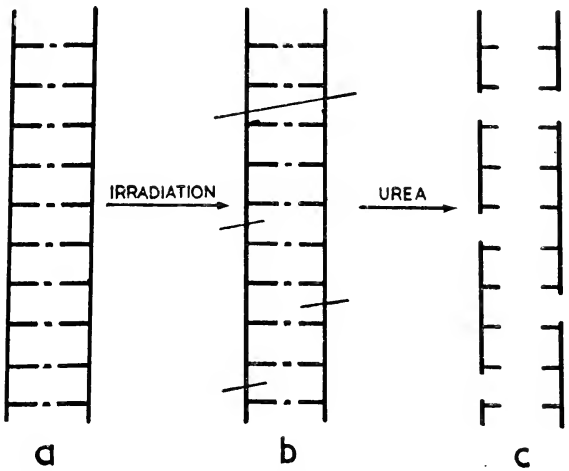


FIG. 1. (Alexander). A diagrammatic representation of the effect of urea on irradiated DNA which shows how breaks hidden in the dimeric structure (b) become apparent in urea solution when all the hydrogen bonds between the chains have been broken.

either in salt or in urea solution. In salt solution the average molecular weight of the sample is hardly changed ($6{\cdot}5 \times 10^6$ to $6{\cdot}0 \times 10^6$), while in urea solution the average molecular weight is more than halved. We have interpreted this as follows: in urea solution the herring sperm DNA molecule is dissociated and the two parts move freely; hence any breaks introduced in any one chain will be revealed by molecular weight measurements. When the material is measured in salt, the two parts do not exist independently and two breaks have to occur in fairly close juxtaposition in the two chains before a break can be detected by a physicochemical measurement. Fig. 1 shows diagrammatically what we believe to happen. Because of the dimer structure of DNA some of these breaks are hidden and are only revealed when the structure is opened out. With light scattering one can make direct measurements of

the molecular weight and determine the number of main chain bonds broken, so that one can get quantitative values for the radiation change. From a series of measurements we find that for every 10–15 electron volts deposited in the nucleic acid (or a G value of more than 5) one break occurs in the main chain if the DNA is irradiated in the solid state. If the DNA is irradiated in solution the G value in the same reaction is of the order of 0·1. In other words, direct action is about 50 times more effective than indirect action for depolymerizing DNA. In the cell where the DNA is present at relatively high local concentration, direct action will be much more important than indirect action as far as depolymerization is concerned. This is relevant to the point made by Prof. Mitchell (see p. 71) with regard to differences in the radiosensitivity of tumours.

Latarjet: Experiments which were carried out on a transforming agent of *Pneumococcus* by Dr. Ephrussi-Taylor and myself are not in agreement with what Dr. Alexander has just said. The purified transforming agent, which is DNA, is tested not by physicochemical properties but by its biological specific activity, and its sensitivity to radiation remains the same whether it has been treated with 5 M urea or not.

Haddow: I don't think that is necessarily an inconsistency.

Alexander: The answer may be that DNA's from different sources don't all behave in the same way towards urea. Herring sperm DNA is dissociated, DNA from thymus is not dissociated until it has received a further treatment (Alexander, P., and Stacey, K. A. (1955), *Nature, Lond.*, **176**, 162).

OXIDATIVE PHOSPHORYLATION IN SOME RADIOSENSITIVE TISSUES AFTER IRRADIATION

D. W. van Bekkum

Medical Biological Laboratory of the National Defence Research Council TNO, Rijswijk

Introduction

Although a considerable number of biochemical studies on irradiated organisms and tissues has been made, information concerning the primary biochemical lesion in irradiated cells is still lacking. So far it seems that the investigation of radiation effects in radiosensitive tissues has been more profitable than the study of radioresistant tissues. Unfortunately our knowledge of the biochemistry of the former is limited compared to that of some of the radioresistant tissues e.g. the liver, the muscles and the brain.

In certain radiosensitive tissues massive cell death occurs within a few hours after irradiation of the animal with a lethal or sublethal dose and these events are bound to be accompanied by a variety of biochemical changes. Therefore the biochemical effects observed in these organs can only be interpreted when the cytological effects are registered as well. Biochemical changes that can be demonstrated before structural damage to the cells becomes apparent are of the greatest interest, because these may be expected to be closely related to or even identical with the primary biochemical lesion. This does not infer however that structural alterations should necessarily always be preceded by some enzymatic disturbance.

The nuclear functions of DNA synthesis and mitosis have been found to be extremely sensitive to ionizing radiation. However, the inhibition of these processes is not limited to radiation and there is no convincing evidence as yet to justify

identification of the disturbance of DNA synthesis with the primary biochemical lesion. Since synthetic processes in the nucleus are generally considered to be dependent on energy-generating reactions which occur in the cytoplasm, it was thought possible that the disturbance of nuclear functions which occurs after irradiation might be the result of damage to biochemical reaction systems outside the nucleus. The cytoplasmic reaction chains by which energy is produced in a transportable form, e.g. ATP, are chiefly anaerobic glycolysis and the oxidations performed by way of the citric acid cycle. In terms of the production of high energy phosphate bonds, the latter are by far the most important.

The oxidative phosphorylations occur in the mitochondria and these cytoplasmic structures have received relatively little attention from radiobiologists. The older literature contains some reports on morphological changes in mitochondria which were observed shortly after irradiation of plant and animal cells (Nadson and Rochlin, 1934; Hirsch, 1931; Colwell, 1935). The changes described are presumably non-specific but this applies to most of the radiation-induced nuclear changes as well. Several of the authors claim that the mitochondrial effects precede the morphological changes which occur in the nuclei. On the other hand, Trowell (1952) in a recent investigation of the effect of radiation on lymphocytes has reported only minor changes of the mitochondria.

In 1952 studies were initiated in this laboratory with the object of collecting information on the effects of ionizing radiation on the oxidative phosphorylations. In preliminary experiments with mitochondria from various tissues, a decreased phosphorylation was observed in rat spleen mitochondria after total body irradiation (van Bekkum *et al.*, 1953). Most of our subsequent work has been carried out with rat spleen mitochondria but this effect has also been demonstrated in mitochondria from mouse spleen and rat thymus. A depression of oxidative phosphorylation in rat spleen mitochondria after total body X-irradiation has also been described by Potter and Bethel (1952) but their paper does not

include detailed results. Maxwell and Ashwell (1953) have reported a decrease in oxidative phosphorylation of mouse spleen mitochondria at 1–7 days after a lethal dose of total body irradiation. The cellular composition of the spleen has undergone radical changes at the time of their studies, so that the results do not throw much light on the significance of the disturbance with regard to the initial radiation effect.

Most of our own observations have been made 4 hours or less after irradiation and considerable attention has been paid to simultaneously occurring cytological effects. Part of the results, which have already been published, will be reviewed briefly below.

Methods

To enable the reader to evaluate our data, a few remarks on the methods employed will be made. The animals—rats in most of the experiments—were irradiated with penetrating X-rays (H.V.L.: 1·8 mm. Cu, dose rate 45 r/min.) under conditions of maximum backscatter. The mitochondria were isolated by differential centrifugation of the homogenates in 0·25 M sucrose solution. Generally the mitochondria were washed twice. The preparations thus obtained from spleen and thymus contain a certain amount of impurities, part of which consists of small nuclear fragments. With the various homogenating techniques it has not been possible to disrupt the majority of the cell membranes without simultaneously breaking a significant number of nuclei. In view of the fact that most of the cells of these tissues have only a narrow brim of cytoplasm surrounding a large nucleus, this may be expected. Oxidative phosphorylation has been measured in a medium containing succinate or α-ketoglutarate as a substrate. Adenylic acid was added to provide phosphate acceptors, and hexokinase plus glucose were employed as a trap for the terminal phosphate group of ATP. $MgCl_2$, fluoride, ethylenediamine tetracetate and cytochrome *c* were present in the system. DPN was added when α-ketoglutarate

was used as a substrate, because washing causes a deficiency of DPN in these mitochondria. Incubation was usually carried out at 37°C for 24 minutes in oxygen. In recent experiments the gas phase was air, which did not make any difference. For a detailed description of these methods reference is made to previous papers (van Bekkum *et al.*, 1954; van Bekkum and Vos, 1955).

It should be pointed out that the properties of spleen and thymus mitochondria are not identical with those of liver mitochondria. Morphological differences have been observed: spleen mitochondria are smaller and of less uniform size. The number of mitochondria per cell is distinctly smaller in spleen and thymus than in liver, and accordingly the yield of mitochondria from the former tissues is much smaller. The P/O ratio of spleen mitochondria is about 1 when succinate is oxidized. Under the same conditions the P/O ratio of liver mitochondria is usually about double that value. The P/O ratio of thymus mitochondria was found to be well below 1. Finally the phosphorylating activity of spleen mitochondria may vary considerably between individual rats. This is probably partly due to the marked variation of the cellular composition of the spleen in rats. It has in fact been shown that mitochondria isolated from spleens, in which the erythropoiesis has been greatly stimulated, display an increased phosphorylation as well as elevated P/O ratios (van Bekkum, 1955*a*). In order to minimize the effect of these variations, the mitochondria were prepared from the pooled spleens or thymus glands of 2–4 rats. Furthermore, in every experiment, control and irradiated tissues were handled simultaneously.

Results

In our earlier experiments the relatively large dose of 1100 r of total body irradiation was administered; 4 hours thereafter the rats were killed and the isolation of the spleen mitochondria was started. A significant depression of the phosphate uptake was present both when succinate and α-ketoglutarate

were used as substrate (Table I). In the experiments with succinate the oxygen uptake was depressed to a less extent than the phosphate uptake, which resulted in a decreased

Table I

Oxidative Phosphorylation of Rat Spleen Mitochondria 4 Hours after Irradiation (1100 r.)*

	Phosphate uptake μ*mole/mg.N*	O_2 *uptake* μ*atoms/mg.N*	*P/O ratio*
Substrate: succinate 0·01 M			
controls (18)	32·7±7·0	28·9±3·8	1·14±0·19
irradiated (19)	17·1±5·0	22·8±4·8	0·82±0·23
P of difference	< 0·001	< 0·001	< 0·001
Substrate: α*-ketoglutarate* 0·01 M			
controls (7)	34·9±6·8	19·7±4·0	1·82±0·44
irradiated (6)	20·9±7·4	13·0±2·2	1·61±0·36
P of difference	< 0·01	< 0·01	< 0·2

* Figures represent means ± S.D. Figures between brackets indicate number of experiments.

P/O ratio. With α-ketoglutarate the decrease in oxygen uptake was more pronounced, and the decrease in the P/O ratio was not statistically significant in this relatively small series of observations (van Bekkum *et al.*, 1954).

Effect of radiation dose

It was soon realized that the above experiments had been performed on a tissue which contained large numbers of dead and degenerating cells. Therefore the effect of the radiation dose on the disturbance of the oxidative phosphorylation, as well as on the amount of nuclear degeneration, was studied. The interval between radiation and the killing of the rats was kept at 4 hours. Doses down to 300 r were found to cause a marked decrease in the phosphate uptake of both spleen and thymus mitochondria. The minimal effective dose appeared to be about 100 r in spleen and 50 r in the case of thymus mitochondria. The histological sections of these thymus glands, after dosage with 50 r, showed definite changes at 4 hours.

These consisted of pyknosis, fragmentation and vacuolization of the nuclei of a small number of thymocytes. The percentage of degenerated nuclei was 3·7 and 8·9 in two representative sections against 1·0 and 1·7 in sections of control glands. The number of mitotic figures was decreased by about 50 per cent in the irradiated group. This amount of nuclear degeneration seems rather small to explain the observed changes of the mitochondrial functions. It was concluded that the oxidative phosphorylation in these tissues represents a cytoplasmic function the radiosensitivity of which is comparable to that of the nuclei.

The interval between irradiation and the appearance of the effect

Because of the possible relation between nuclear metabolism and oxidative phosphorylation it seemed of interest to investigate whether the disturbance of the latter becomes discernible prior to or after the beginning of nuclear degeneration. This was studied on spleen tissue after a total body dose of 700 r (van Bekkum and Vos, 1955). Measurements were made at 15 minutes, 30 minutes, 1, 2 and 4 hours after irradiation. The earliest significant depression of phosphate uptake was found at 2 hours (Fig. 1), when oxygen consumption was only slightly depressed. The histological findings may be summarized as follows: from 15 minutes up to 2 hours after irradiation the mitotic frequency was greatly diminished, at 4 hours some reappearance of mitotic figures was noted. Signs of nuclear degeneration were absent or dubious at 15 and 30 minutes after irradiation. After 1 hour early stages of nuclear degeneration became clearly discernible in a few cells. At 2 hours nuclear degeneration was present in 10–20 per cent of the lymphocytes and at 4 hours the majority of the cells showed pyknosis or nuclear fragmentation. Nuclear damage could thus clearly be observed before a significant decrease of phosphorylation was demonstrable.

This, however, cannot be taken as proof that nuclear degeneration precedes the mitochondrial lesion. Because individual

cells are inspected, the detection of cytological change is much more sensitive than the estimation of oxidative phosphorylation which has to be carried out on the pooled mitochondria from all the cells in a tissue. When, on the other hand, the total number of damaged nuclei is compared with the overall change of phosphorylation it may be stated that the development of nuclear and mitochondrial damage runs roughly parallel. In recent experiments results were obtained which

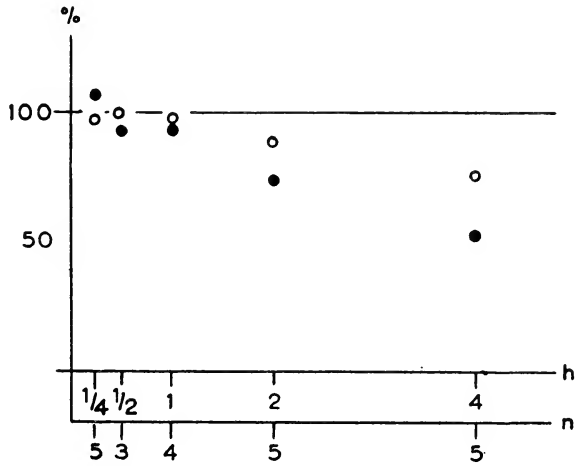


FIG. 1. Oxidative phosphorylation of rat spleen mitochondria at various periods after total body irradiation (700 r). Substrate: succinate. Abscissae: hours after irradiation. Ordinates: percentage of corresponding control values. White circles: oxygen uptake. Black circles: phosphate uptake. n: number of experiments.

suggest that a biochemical defect in spleen mitochondria is present as early as 30 minutes after total body irradiation, when the only demonstrable cytological effect is the inhibition of mitosis. These experiments will be described in one of the following sections.

Tissue specificity of the effect

A rough survey has been made of the occurrence of the disturbance of oxidative phosphorylation after total body

irradiation in various tissues. The postirradiation interval was 4 hours in all cases because after longer periods secondary effects may be expected. So far, the disturbance has only been found in spleen and thymus mitochondria. Results with liver, regenerating liver and a number of transplantable mouse tumours were negative even after doses of several thousands of r. Intestinal mucosa and bone marrow did not yield satisfactory mitochondrial preparations so that these tissues could not be studied.

To investigate whether the effect in the mitochondria occurs also after local irradiation of the spleen, the exteriorized spleen was irradiated with the rest of the body of the rat shielded. A depression of oxidative phosphorylation was found, which was comparable to that observed after total body irradiation, and therefore the effect is at least chiefly due to the action of X-rays on the spleen cells directly. As to the nature of the cells containing these sensitive mitochondria, evidence has been presented that the mitochondria from both lymphoid and erythropoietic cells are susceptible to irradiation (van Bekkum, 1955*a*).

It has not been possible to reproduce the effect of radiation on the mitochondria *in vitro*, even with doses as large as 20,000 r. Similar negative findings have been reported by Potter and Bethel (1952) and by Ord and Stocken (1955). In our opinion these negative *in vitro* findings carry little weight, since it is not possible to imitate even remotely the conditions which exist inside the cell.

On the nature of the mitochondrial defect

The possibility has been considered that decreased phosphorylation after irradiation might be artificially induced during the isolation process, as a result of some unknown and completely unrelated change of the irradiated tissues, e.g. an alteration of the viscosity of the homogenates. Therefore an attempt was made to measure the rate of phosphorylation *in vivo* by the use of radioactive phosphate. Rats were injected intravenously with labelled inorganic phosphate

and after a suitable interval their spleens were excised under Nembutal anaesthesia and immediately frozen in liquid air. The frozen tissue was homogenized in perchloric acid and the nucleotides were adsorbed from the neutralized perchloric acid extract on charcoal. Elution was carried out with pyridine solutions and AMP, ADP and ATP fractions were separated by ion exchange on Amberlite IRA 400 according to a modification of the method described by Cohn and Carter (1950).

The specific activity of the ATP of the tissue can thus be estimated, but the turnover rate of the phosphate groups of ATP cannot be measured because no method is available to determine the specific activity of the intracellular inorganic phosphate which is supposedly the immediate precursor of these groups. Therefore the specific activity of the total inorganic phosphate was measured, and the specific activity of the labile phosphate groups of ATP was expressed relative to the former value (relative specific activity). Some of the results obtained 4 hours after irradiation with a dose of 700 r are summarized in Table II.

Table II

THE EFFECT OF TOTAL BODY IRRADIATION ON THE INCORPORATION OF LABELLED INORGANIC PHOSPHATE IN RAT SPLEEN ATP *in vivo**

	ATP specific activity: arbitrary units	*ATP relative specific activity*
Controls	$10{\cdot}9 \pm 1{\cdot}5$	$0{\cdot}66 \pm 0{\cdot}06$
4 hours after 700 r total body dose	$7{\cdot}9 \pm 1{\cdot}3$	$0{\cdot}56 \pm 0{\cdot}04$
P of difference	$< 0{\cdot}001$	$< 0{\cdot}001$

* The figures in the table represent means of 10 observations ± S.D.

The specific activity of ATP as well as the relative specific activity of its labile phosphate groups were found to be significantly depressed. Although this cannot be considered to prove the existence of a disturbance of phosphorylating reactions *in vivo* the data are at least in complete agreement with the observations on isolated mitochondria. It is noteworthy that no difference was observed in ATP content

between the control and the irradiated groups. Similar results have been obtained with this technique in thymus glands.

An increase of ATPase activity in spleen homogenates after total body irradiation has been reported by several authors (Ashwell and Hickman, 1952; Dubois and Petersen, 1954) and the question arose whether the depression of oxidative phosphorylation might be related or even secondary to this phenomenon. A more detailed investigation showed that the increase of ATPase activity appears several hours after the disturbance of oxidative phosphorylation has become established. Furthermore, normal ATPase activities were found in mitochondria that exhibited a markedly decreased phosphorylating capacity (van Bekkum, 1955*b*). Finally Petersen, Fish and Dubois (1955) reported that the ATPase effect is absent after irradiation of the exteriorized spleen exclusively and it should therefore be classified among the secondary radiation effects.

The decrease in anaerobic glycolysis which was described by Hickman and Ashwell (1953) in mouse spleen homogenates one or more days after a total body irradiation, was found to be absent at 2 and 4 hours after irradiation, when oxidative phosphorylation was severely depressed (van Bekkum, 1955*a*). It was concluded that different mechanisms are probably involved in the development of these biochemical changes.

At present we are engaged in an investigation of the various phosphorylative steps in spleen mitochondria from irradiated rats. So far, some interesting data have been obtained on the relation of cytochrome *c* to the depression of oxidative phosphorylation. When cytochrome *c* is omitted from the succinate reaction system both phosphate uptake and oxygen consumption drop about 50 per cent in the control preparations. In other words the addition of cytochrome *c* causes an appreciable stimulation (cytochrome *c* effect). This cytochrome *c* effect is found to be consistently increased after irradiation (Table III).

Even at 30 minutes after irradiation (700 r) a slightly increased cytochrome *c* effect has been observed. If the

values obtained at 30 minutes and at 1 hour after irradiation are pooled the differences between irradiated and control preparations are found to be statistically significant both in the case of phosphate and oxygen uptake. This demonstrates an alteration of a mitochondrial function at a time when nuclear degeneration is absent or limited to a few cells only. Table III further shows that the cytochrome *c* effect increases rapidly after the first hour, and that at 4 hours after irradiation the very low phosphate uptake of the mitochondria is more markedly stimulated by added cytochrome *c* than is the oxygen consumption. In this case cytochrome *c*

Table III

INFLUENCE OF TOTAL BODY IRRADIATION (700 r) ON THE CYTOCHROME *c* EFFECT IN SPLEEN MITOCHONDRIA*

Hours after irradiation	% *stimulation by cytochrome c*†			
	Phosphate uptake		O_2 *uptake*	
	control	*irradiated*	*control*	*irradiated*
0·5	65 (4)	77 (4)	82 (4)	95 (4)
1	63 (4)	86 (4)	66 (3)	91 (3)
2	90 (4)	180 (3)	92 (4)	165 (4)
4	82 (2)	362 (2)	84 (2)	172 (2)

* Mean values are presented, the number of experiments is given between brackets.
† Stimulation is expressed in percentage of the values obtained in the absence of cytochrome *c*. Substrate : succinate; concentration of cytochrome *c*: 28×10^{-5}M. The mitochondria were washed twice with sucrose.

nearly doubles the P/O ratio. These results suggest that in spleen mitochondria a relative cytochrome *c* deficiency develops very shortly after irradiation.

It was of course essential to know whether this reflects an interference by radiation with some part of the cytochrome system. It was not inconceivable that some radiation-induced structural alteration of the mitochondria might cause a leakage of cytochrome *c* during the isolation, in which case washing of the mitochondria might be expected to increase this leakage. In previous experiments with normal spleen mitochondria it was found that repeated washing causes a deficiency of DPN, which could be demonstrated in the α-ketoglutarate system.

To test the possibility of the occurrence of a similar leakage

of cytochrome *c* being aggravated as a result of irradiation, the cytochrome *c* effect was estimated at 2 hours after irradiation in unwashed mitochondria. A comparison of the results (Table IV) with those obtained on twice washed

Table IV

EFFECT OF THE ADDITION OF CYTOCHROME *c* AND OF DPN ON OXIDATIVE PHOSPHORYLATION OF UNWASHED SPLEEN MITOCHONDRIA FROM CONTROL AND IRRADIATED RATS (2 HOURS AFTER 700 r)*

	Phosphate uptake		*O_2 uptake*	
	control	*irradiated*	*control*	*irradiated*
% stimulation† by cytochrome *c* in succinate system	119	360	102	195
% stimulation† by DPN in α-ketoglutarate system	8	5	7	13

* Values represent means of 3 experiments; 4 control and 4 irradiated rats were used per experiment.

† Stimulation is expressed in percentage of the values obtained in the absence of the stimulating substance. Concentration of cytochrome *c*: $2 \cdot 8 \times 10^{-5}$M; DPN: 4×10^{-4}M.

mitochondria (Table III) shows that the cytochrome *c* effect is not increased by washing. Therefore this simple explanation cannot be applied. It is also to be noted that irradiation does not result in an increased leakage of DPN.

Conclusions

At present we are still far from a complete understanding of the biochemical changes that take place in spleen mitochondria after irradiation. However, it seems fairly certain that these changes occur shortly after irradiation and that the cytochrome system is somehow involved. It is tempting to connect the fact that irradiation affects this part of the respiratory chain with the well-known observations on the oxygen effect in irradiation experiments with living organisms.

In this connection it is of interest that Laser (1954) has also suggested the involvement of the cytochromes in the biochemical effect of radiation. However, much additional information is required before the nature of this involvement can be more accurately defined.

REFERENCES

ASHWELL, G., and HICKMAN, J. (1952). *Proc. Soc. exp. Biol., N.Y.*, **80**, 407.
BEKKUM, D. W. VAN (1955*a*). Radiobiology Symposium, p. 201. London: Butterworth.
BEKKUM, D. W. VAN (1955*b*). *Biochim. biophys. acta*, **16**, 437.
BEKKUM, D. W. VAN, JONGEPIER, H. J., NIEUWERKERK, H. T. M., and COHEN, J. A. (1953). *Trans. Faraday Soc.*, **49**, 329.
BEKKUM, D. W. VAN, JONGEPIER, H. J., NIEUWERKERK, H. T. M., and COHEN, J. A. (1954). *Brit. J. Radiol.*, **27**, 127.
BEKKUM, D. W. VAN, and VOS, O. (1955). *Brit. J. exp. Path.*, **36**, 432.
COHN, W. E., and CARTER, C. E. (1950). *J. Amer. chem. Soc.*, **72**, 4273.
COLWELL, H. A. (1935). The Method of Action of Radium and Alpha-rays in Living Tissue. London: Oxford University Press.
DUBOIS, K. P., and PETERSEN, D. F. (1954). *Amer. J. Physiol.*, **176**, 282.
HICKMAN, J., and ASHWELL, G. (1953). *J. biol. Chem.*, **205**, 651.
HIRSCH, G. C. (1931). *Arch. EntwMech. Org.*, **123**, 792.
LASER, H. (1954). *Nature, Lond.*, **174**, 753.
MAXWELL, E., and ASHWELL, G. (1953). *Arch. Biochem.*, **43**, 389.
NADSON, G. A., and ROCHLIN, E. J. (1934). *Protoplasma*, **20**, 31.
ORD, M. G., and STOCKEN, L. A. (1955). *Brit. J. Radiol.*, **28**, 279.
PETERSEN, D. F., FISH, F. W., and DUBOIS, K. P. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 394.
POTTER, R. L., and BETHEL, F. N. (1952). *Fed. Proc.*, **11**, 270.
TROWELL, O. A. (1952). *J. path. Bact.*, **64**, 687.

DISCUSSION

Loutit: It is very heartening to me to see this correlation between biochemical and histological findings, for so rarely do we have the two together. I would like to ask whether the cytologist or histologist was always doing his work unseen as it were; did he know what he was looking at, whether it was a half-hour, two-hour or four-hour section; or could he have been biased by a previous knowledge of the time at which the section was taken?

Van Bekkum: The histologist, Dr. O. Vos, only knew that some of the sections were taken at say 30 minutes and others 1, 2 or 4 hours after irradiation, but he did not know which one. On the basis of nuclear changes he could not differentiate between 15 minutes after irradiation, 30 minutes after irradiation and control sections, but he could differentiate between them because of the decrease of mitotic figures in the slices from irradiated rats.

Koller: I have seen lymphocytes, irradiated with quite small doses, which are undergoing nuclear disintegration similar to that described by Dr. Van Bekkum. The degeneration of the lymphocytes in extremely high numbers cannot be explained by the old theory that the cells have gone through mitosis after irradiation and die because of chromosome fragmentation. That raises a very important point which is included

in Dr. Van Bekkum's presentation: have we in the lymphocytes a different system as far as sensitivity towards radiation is concerned?

Loutit: In our laboratory we have seen, in other tissues besides lymph tissues, the break-up of cells before mitotic division, with a dose of about 150 r. I do not think it is confined to this particular system.

Mitchell: This acute cytolysis is seen in some of the cells of the highly radiosensitive and radio-curable basal-cell carcinoma of the skin; even after 50 r within about 45 minutes areas of liquefaction have been seen.

Holmes: It may be relevant to Dr. Koller's question to describe an experiment we carried out for Dr. Trowell. He asked us to estimate the DNA synthesis in lymph glands where he was quite sure there was no mitosis. We found more uptake of ^{32}P into the DNA fraction than one would expect in a resting tissue. It seemed just possible that even if no division was occurring the DNA was undergoing more change than it does in other tissues.

Brachet: Dr. Van Bekkum, is there any contamination of nuclear material in your mitochondria, for instance is there any DNA? Could it be that, when there is this destruction of nuclei, material originating from degenerating nuclei might mix with your mitochondrial fraction? According to Allfrey and Mirsky (1955, *Nature, Lond.*, **176**, 1042), isolated thymus nuclei might be the site of some sort of oxidative phosphorylation. It may be specially important, if that is true, to know whether you have any nuclear contamination or not.

Van Bekkum: I cannot believe that it is possible, as Mirsky claims, to completely separate the nuclei from the cytoplasm in a tissue such as the thymus. As far as I can remember, Allfrey and Mirsky did not demonstrate phosphorylation in isolated liver cell nuclei, which are much easier to obtain, and in my opinion that is what should be done.

Brachet: I quite agree with you that that should also be done.

Van Bekkum: I am not convinced that the experiments of Allfrey and Mirsky have proved beyond doubt that nuclei or nuclear fragments are capable of oxidative phosphorylation. I am sure that even our control preparations do contain a certain amount of nuclear fragments. I have not been able to avoid that by varying the methods of homogenization and differential centrifugation. Therefore we have spent a considerable amount of time on the *in vivo* studies with radioactive phosphate and we have interpreted the results as supporting our *in vitro* findings.

Howard: Coming back to the correlation of the histological effects in the spleen, it is clear that lymphocytes can be killed by very small doses of radiation. The dead cells take a little time to appear in the tissue, but I do not think we have to assume that there is no change in the cell population until the dead cells appear. There are changes taking place as soon as there is a mitotic arrest. You only have to suppose that the function of the mitochondria has something to do with the cell cycle, or perhaps even that cytochrome *c* is bound up with the cell cycle in some way, and you will expect that an hour or two hours after irradiation, when there has been a blocking of cells entering mitosis, this might be reflected in the activity of the mitochondria. So I think these histo-

logical changes, even if we are not prepared to accept a lot of cell death within the first two hours, are still very significant indeed to the interpretation of the results.

Van Bekkum: What time do you suppose it takes for a lymphoid cell to go through mitosis?

Howard: I think the cycle must be fairly rapid, of the order of a few hours.

Van Bekkum: Well, in our spleen tissues, at least, the number of mitotic figures is very low compared to the total number of cells, of the order of 2 or 3 per thousand. I don't think that the explanation you suggest can cover this if you consider what a small number of cells is moved by it per hour.

Howard: The whole cycle probably takes a few hours, but there may be a very short time spent in division. I would imagine that cell turnover is quite rapid.

de Hevesy: There is a large fraction of lymphocytes having a very long life-time, as found by Ottesen, the existence of which may explain Dr. Van Bekkum's finding.

Laser: Dr. Van Bekkum, I take it that you agree that irradiation of *isolated* mitochondria has no effect on oxidative phosphorylation. In this connection, I would like to mention one result which has rather surprised us. Dr. Slater and I have measured the actual oxidative phosphorylation during radiation. The system was so adapted that within six minutes, during which we applied 30,000 r, we could measure the disappearance of α-ketoglutarate and the phosphorylation. There was no significant effect at all, which means that these processes went on undisturbed during the actual application of 30,000 r to the isolated material.

Van Bekkum: We have done the same sort of thing with similarly negative results. Did you add cytochrome *c* to the system during the measurement of phosphorylation?

Laser: Yes.

Van Bekkum: So did we.

Popjak: Dr. Van Bekkum, have you an explanation for the rather low P/O ratio in this particular type of mitochondria? It seems to me that from spleen and thymus you are getting a P/O ratio of barely over 1. One normally gets higher P/O ratios with liver mitochondria.

Van Bekkum: I only know that Dr. Slater has also found, in the case of heart mitochondria, a rather low P/O ratio of about 1 in the presence of succinate. I don't know of any explanation for that.

Loutit: With this level of dose the period of two hours seems to me a very critical one. There is also evidence, in addition to histological and biochemical evidence, for cell death at this time. The ordinary sodium pump mechanism has been shown to break down at this time and sodium leaks into the cell and potassium leaks out. Furthermore, we have already discussed the loosening of the enzyme systems, that enzymes are getting into places where they should not be, which might be the preliminary stage for this final blow-up and deathblow to the cell.

THE EFFECTS OF EXTRANEOUS AGENTS ON CELL METABOLISM*

H. A. KREBS

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

THE question has been raised in the preceding contributions whether or not a study of the effects of ionizing radiation on isolated enzyme systems may be expected to solve some of the problems of radiation biology. I would like to offer comments on this question.

I think it is correct to say that all effects on living cells of radiation, and of extraneous agents generally, are brought about through interference with some chemical substance in the cell. If this substance is an enzyme, the effect of the external agent (probably always a reduction of catalytic activity) may cause a significant disturbance in cell metabolism, but this is not necessarily the case. Whether a change of enzyme activity is of major consequence depends on whether the enzyme concerned plays a rôle in the rate control of metabolism.

Pacemaker reactions as vulnerable stages of cell metabolism

The analysis of the circumstances which control the rates of metabolic processes shows that the amount of enzyme present is by no means always the controlling factor. It is true that

* Abbreviations used:

DPN	diphosphopyridine nucleotide
$DPNH_2$	reduced diphosphopyridine nucleotide
ATP, ADP	adenosine triphosphate, adenosine diphosphate
GTP, GDP	guanosine triphosphate, guanosine diphosphate
TPP	thiamine pyrophosphate
P	inorganic orthophosphate
CoA	coenzyme A

the amounts of enzyme and substrate determine the maximum rate of the process but maximum rates are exceptional under physiological conditions. The limiting factor is usually (though not always) the amount of substrate. The fact that intermediate products generally do not accumulate shows that the substrates of the intermediary enzymes are removed as rapidly as they are formed. The average half-life of the acids of the tricarboxylic acid cycle in a rapidly respiring tissue is of the order of a few seconds (Krebs, 1954). Thus the amount of enzyme in the tissue is sufficient to deal with the intermediate as soon as it arises; in other words, the amount of available substrate is the factor limiting the rate at which many intermediary steps proceed. Hence a partial destruction of an enzyme does not necessarily upset cell metabolism.

It is obvious, however, that this cannot apply to all steps of metabolism. There are some reactions, small in number by comparison, where the rates depend on factors other than the amounts of enzyme or substrate. These are the "pacemakers" of metabolism (Krebs, 1956).

The pacemaker reactions are the steps of metabolism which are especially vulnerable to extraneous agents, because any decrease in activity is liable to show itself in a diminished overall rate of metabolism. Pacemakers are therefore the enzyme systems towards which the study of the effect of extraneous agents should be primarily directed. I would like to elaborate these considerations on some examples, taken mainly from the field of energy transformations.

Effects of extraneous agents on anaerobic glycolysis

The anaerobic glycolysis is known to involve some twelve separate steps. The factors which control the overall rate of glycolysis and adjust the rate to the physiological energy requirements are by no means fully known but there are two steps which have been identified as pacemakers. The first is the hexokinase reaction (LePage, 1950) which probably initiates all major metabolic reactions of glucose, such as the

anaerobic fermentation, the complete oxidation and the transformation into glycogen, pentoses, fat, amino acids, or other cell constituents. The second is the triosephosphate dehydrogenase systems. This is a complex reaction in which, apart from triosephosphate and the catalysts, at least three other reactants—DPN, ADP and orthophosphate—are required:

$$\left.\begin{matrix}\text{triosephosphate} + \text{DPN} \\ + \text{ADP} + \text{P}\end{matrix}\right\} \rightleftharpoons \left\{\begin{matrix}\text{phosphoglycerate} + \text{DPNH}_2 \\ + \text{ATP}\end{matrix}\right.$$

As the coupling between the dehydrogenation of the substrate and the synthesis of ATP is obligatory the reaction cannot take place unless ADP and phosphate are available. The concentrations of these two substances are in fact assumed to be the rate-limiting factors (Lardy and Wellman, 1952). The concentrations are bound to vary with the functional state of the cell, especially with the rate of the expenditure of ATP. Since oxidative phosphorylation removes ADP and phosphate, respiration must inhibit glycolysis, an interpretation of the "Pasteur effect" first suggested by Lynen (1941) and Johnson (1941).

There must be additional pacemakers of anaerobic glycolysis which decide the fate of glucose-6-phosphate. This intermediate is placed at one of the branching points of metabolism. Apart from giving lactate through glycolysis it can be transformed into glycogen or oxidized to phosphogluconate and pentose phosphate, but nothing definite is known about the factors controlling the choice between these alternatives.

Many of the inhibitors of glycolysis are substances which react with the two known pacemaker enzymes. Thus bromo- and iodoacetate inhibit triosephosphate dehydrogenase, by combining with the sulphydryl group of glutathione, the prosthetic group of the enzyme. The hexokinase reaction is inhibited by various hexosephosphates, in particular by the product of the reaction, glucose-6-phosphate (Weil-Malherbe and Bone, 1951), and by L-sorbose-1-phosphate (Lardy, Wiebelhaus and Mann, 1950). This inhibition is non-competitive. The two pacemakers are also the points of attack of

glyceraldehyde, a powerful inhibitor of glycolysis (Mendel, 1929; Rudney, 1949). L-Glyceraldehyde is transformed in glycolysing material into L-sorbose-1-phosphate under the influence of aldolase. D-Glyceraldehyde probably inhibits triosephosphate dehydrogenase (Needham, Siminovitch and Rapkine, 1951).

Whilst pacemakers are more vulnerable to extraneous agents than non-pacemaker reactions, the latter are not immune to inhibitors; but a substantial proportion of a non-pacemaker must be inactivated before the overall rate is affected. An inhibitor of glycolysis which interferes with a non-pacemaker is fluoride. It inhibits enolase, the enzyme which converts 2-phosphoglycerate to enolphosphopyruvate (Meyerhof and Kiessling, 1933; Lohmann and Meyerhof, 1934). However, fluoride also inhibits other enzymes, in particular those dependent on magnesium ions as a cofactor, such as adenosine triphosphatase and some phosphate-transferring enzymes. It is by no means established that the inhibition of glycolysis is solely due to the inactivation of enolase.

Effects of extraneous agents on cell respiration

When energy is released by the oxidation of carbohydrate, fat and amino acids, there are over a hundred identifiable intermediate steps, only a few of which are pacemakers. The non-accumulation of intermediates indicates that those steps which initiate the oxidation of a substrate, i.e. the reaction between substrates and their dehydrogenase, must be among the pacemakers of respiration. Once the oxidation has been started, most of the subsequent reactions, leading to complete combustion, follow at the pace set by the initiating step, owing to the excess of enzymes dealing with the intermediary products. The initiating reactions also decide which substrate among a mixture is attacked preferentially—whether carbohydrate, fatty acid or amino acids serve as a source of energy. In addition, pacemakers are expected at two other types of stages of the oxidative metabolism, at those where the

rate of oxygen consumption is determined, and at those where, after a partial degradation, more than one pathway is open; in other words, where the pathways of metabolism can branch.

Before I discuss the effect of inhibitors on the different types of pacemaker reactions, I must make reference to the mechanism by which the rate of oxygen consumption is controlled. One of the decisive factors is the rate at which hydrogen atoms or electrons travel from reduced DPN, via flavoprotein and cytochrome *c*, to molecular oxygen. Unless the catalysts of the electron carrier chain are in the oxidized form the substrates cannot be attacked. Further, the transport of electrons under physiological conditions is coupled with the synthesis of ATP from ADP and orthophosphate ("oxidative phosphorylation"):

$$\left.\begin{matrix} DPNH_2 + \tfrac{1}{2}O_2 \\ ADP + P \end{matrix}\right\} \rightarrow \left\{\begin{matrix} DPN + H_2O \\ ATP \end{matrix}\right.$$

This coupling appears to be obligatory. Hence the rate of oxygen consumption reaches a maximum value only if ADP and orthophosphate are present above certain critical concentrations and it falls when the concentration of the phosphates falls below the critical level. In most tissues the physiological concentrations of ADP and P are generally below the critical level. The rate of oxygen consumption therefore depends on the rate at which ATP is split in the tissue to ADP and P, i.e. on the rate at which energy is spent. It is thus evident that the component reactions of oxidative phosphorylation are pacemakers.

The following examples show, for the case of cell respiration, that extraneous agents interfere with pacemakers rather than with other intermediary steps of metabolism. Inhibitors of respiration fall into three main classes, according to the type of pacemaker which they inhibit. Substances of Class I inhibit rate of oxygen consumption because they interfere with electron transport. Class II interferes with the initiating reaction of respiration and therefore affects the type of substrate which is oxidized. Class III interferes with the

mechanism controlling the branching points of metabolism and can therefore divert metabolism from one pathway to another.

Inhibitors belonging to Class I are hydrocyanic acid, azide, carbon monoxide or sulphide which stop the electron transport from iron porphyrin to molecular O_2. Another example is antimycin A which combines with an unidentified component of the transport chain between dehydrogenase and cytochrome *c* and therefore inhibits the oxidation of the substrate by molecular oxygen whilst not preventing ferricyanide from acting as an electron acceptor (Potter and Reif, 1952; Copenhaver and Lardy, 1952).

Inhibitors of Class II interfere at the dehydrogenase level. If the inhibitor is specific for one dehydrogenase, or one type of dehydrogenase, it does not necessarily alter the overall rate of oxidation because other substrates can take the place of that which is prevented from reacting. Thus, cells exposed to malonate which can no longer oxidize succinate at the usual rate may still consume oxygen at the normal rate, if another substrate, such as fumarate, is available.

The initial step of substrate degradation can be brought about by several different types of reaction. In most cases this is a more or less direct transfer to pyridine nucleotide according to the general formula:

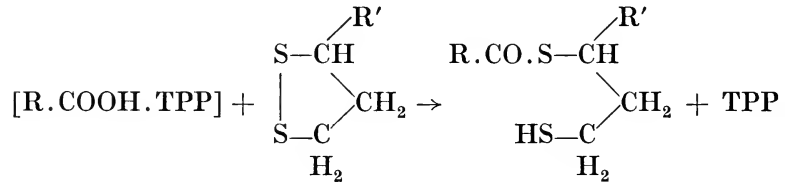
$$\text{(1)}\quad \text{substrate} + \text{DPN} \xrightarrow{\text{dehydrogenase}} \text{oxidized substrate} + \text{DPNH}_2$$

The α-ketonic acids require a more complex mechanism which involves at least six additional cofactors: coenzyme A, α-lipoic acid, cocarboxylase, ADP, GDP and inorganic phosphate. In the case of α-ketoglutarate the following reaction mechanism has been formulated (Gunsalus, 1954). The initial step is taken to be a reaction between α-ketoglutarate and thiamine pyrophosphate (TPP) in which a succinic semialdehyde-TPP-complex is formed and CO_2 liberated:

$$\text{R.CO.COOH} + \text{TPP} \rightarrow [\text{R.COH.TPP}] + \text{CO}_2$$

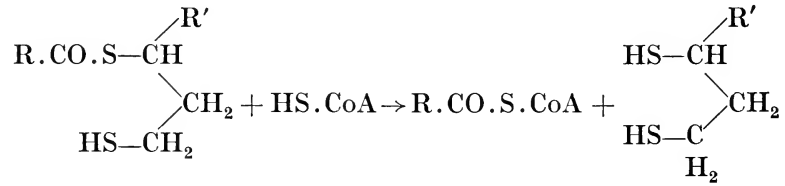
$(\text{R} = \text{COOH.(CH}_2)_2)$

The succinic semialdehyde TPP complex then reacts with the disulphide form of α-lipoic acid in such a manner that (a) the aldehyde group of the TPP complex is oxidized to the corresponding carboxyl whilst the disulphide is reduced to the dimercaptan; (b) the nascent carboxyl and one of the nascent mercaptan groups condense to form succinyl lipoic acid. TPP is regenerated in this reaction:

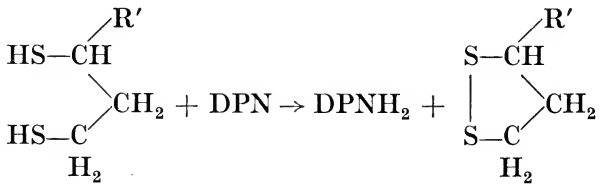


$(R' = COOH.(CH_2)_4)$

The next stage is a transfer of the succinyl group from lipoic acid to coenzyme A, yielding reduced α-lipoic acid and succinyl coenzyme A:



The reduced lipoic acid interacts with DPN under the influence of lipoic acid dehydrogenase to yield a reduced pyridine nucleotide:



Succinyl coenzyme A reacts with GDP and inorganic phosphate to regenerate reduced coenzyme A and to form GTP

and succinate, and GTP and ADP subsequently react to form GDP and ATP:

$$\text{succinyl CoA} + \text{GDP} + \text{P} \rightarrow \text{succinate} + \text{GTP} + \text{CoA}$$

$$\text{GTP} + \text{ADP} \rightarrow \text{ATP} + \text{GDP}$$

Other α-ketonic acids probably react analogously to α-ketoglutarate, at least as far as the reactions with TPP, lipoic acid and coenzyme A are concerned. In the case of pyruvate and possibly other cases, the acyl coenzyme A arising in the

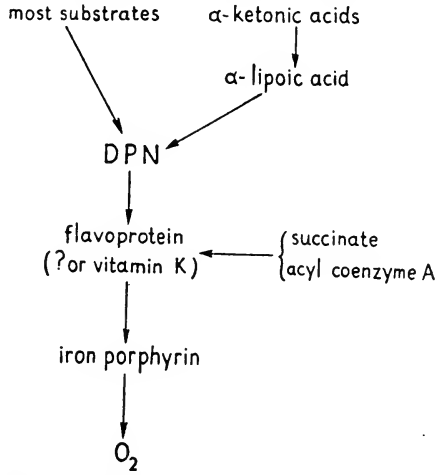


FIG. 1. Pathway of hydrogen transport for different types of substrates.

primary stages is assumed to react with AMP to form acetyl AMP, and subsequently acetate and ATP (Berg, 1955).

Apart from α-ketonic acids there are a few other substrates which do not react according to the common rule (reaction 1). They are succinate, and fatty acids attached to coenzyme A, possibly also some amino acids. In these cases DPN is not involved in hydrogen transport. Instead there is a direct transfer of hydrogen atoms to a flavoprotein, and thence to iron porphyrins. These exceptions arise from the thermodynamic properties of the substrates.

Fig. 1 summarizes the stages in hydrogen and electron

transport from different types of substrates to O_2. It differs in two respects from earlier schemes. One concerns the rôle of vitamin K. Martius (1956) has provided evidence suggesting that vitamin K is an essential link which might replace flavoprotein in some cases. The other concerns the rôle of flavoprotein in hydrogen transport from succinate. That flavoprotein is required in this case has recently been established by Green, Mii and Kohout (1955) and Kearney and Singer (1955).

The differences in the complexity of the various dehydrogenase systems are reflected by differences in their behaviour towards extraneous agents. It has long been known that the oxidation of α-ketonic acids is sensitive to reagents, for example, arsenite (Krebs, 1933), which do not affect other dehydrogenases. Arsenite reacts with SH groups and the specific action of arsenite on the oxidation of α-ketonic acids can be understood on account of the special rôle played by sulphydryl compounds in the dehydrogenation of α-ketonic acids.

The relative simplicity of the succinic dehydrogenase system explains the fact that the oxidation of this substrate is more stable towards environmental changes than that of other substrates. Depriving the tissue of soluble cofactors by washing of minced material with water, inactivates all major dehydrogenase systems except succinic dehydrogenase.

Examples of inhibitors of Class III acting at a branching point of metabolism are agents inducing the formation of ketone bodies in the liver. Among the pathways open to acetyl coenzyme A in liver, there is the condensation with oxaloacetate (i.e. entry into the tricarboxylic acid cycle) or the condensation with another molecule of acetyl coenzyme A [i.e. formation of acetoacetate ("ketogenesis")]. The first requires oxaloacetate as a reactant, and much of the evidence is in accordance with the view that the steady-state level of oxaloacetate is a key factor in the control of ketogenesis.

Agents which reduce the supply of oxaloacetate in the

liver are therefore expected to be ketogenic. This is in fact the case. Malonate, which prevents the conversion of succinate to oxaloacetate, or ammonium chloride which diverts the metabolism of α-ketoglutarate to glutamate are both ketogenic (Recknagel and Potter, 1951; Krebs and Kornberg, 1956).

Effects of extraneous agents on cell activities depending on energy supply

What has been said so far all refers to the reactions supplying energy. Another group of metabolic processes depends on a supply of energy which must generally be available in the form of ATP. To these belong all synthetic processes, (especially the formation of macromolecules from basic units), active transport of solutes, active movement and chemical processes associated with other specific functional activities of the cells. Very little is so far known about the nature of the enzymes concerned with these aspects of metabolism; it is not possible in this field to draw up schemes similar to those representing the energy-supplying reactions.

However, one general feature appears to be shared by many extraneous agents which interfere with processes depending on energy. Although it may not be possible to define the chemical reactions which are obstructed, many effects can be explained by the assumption that the interfering agent is chemically similar to a physiological agent, and that owing to this similarity it occupies the physiological site, thus displacing the physiological agent from its normal position. To quote examples, this mechanism may account for:

(1) The growth inhibition by sulphonamides (which occupy the position of *p*-aminobenzoic acid) and by other antimetabolites like aminopterin (an antifolic acid agent), 6-mercaptopurine (an antipurine agent) and halogen-substituted phenylalanine derivatives (antiphenylalanine agents in protein synthesis).

(2) The anticoagulant effects of dicoumarol which interferes with the conversion of vitamin K into prothrombin.

(3) The action of many drugs, especially the blocking agents. Cholinergic blocking agents (atropine, curare, tetraethyl ammonium ions) are assumed to prevent the attachment of the acetylcholine to the hypothetical receptor site, whilst adrenergic blocking agents (ergotoxin, veratrin) analogously block the adrenergic transmission.

(4) The toxic, or some of the toxic, effects of fluoroacetate which replaces acetate in the formation of citrate and thereby yields fluorocitrate which in turn is a powerful enzyme inhibitor.

In view of the widespread occurrence of this type of interference the idea suggests itself that it might also be responsible for some of the effects of radiation; that the decomposition products of water arising from ionizing irradiation so modify cell constituents that they become noxious and that the noxiousness is due to the similarity to normal constituents.

Conclusions

To sum up, the main thesis put forward in this contribution is the concept that some stages of metabolism in living cells are more vulnerable than others to attack by extraneous agents. The vulnerable stages are those which control the rates of metabolic processes—the "pacemaker" reactions. The enzyme systems responsible for these reactions are expected to work to full capacity under physiological conditions, so that any change in the amount of active enzymes will modify the rate of metabolic processes. In contrast, the enzymes operating at other stages are present in excess of the available substrate, and a partial destruction of the enzymes therefore does not affect the rate of metabolism. Information on the nature and mechanism of action of "pacemaker" reactions is still limited, but the available information for the case of energy transformations confirms that interference by extraneous agents with cell metabolism is more often than not due to interference with pacemaker reactions.

REFERENCES

BERG, P. (1955). *J. Amer. Chem. Soc.*, **77**, 3163.
COPENHAVER, J. H., and LARDY, H. A. (1952). *J. biol. Chem.*, **195**, 225.
GREEN, D. E., MII, S., and KOHOUT, P. M. (1955). *J. biol. Chem.*, **217**, 551.
GUNSALUS, I. C. (1954). *Fed. Proc.*, **13**, 715.
JOHNSON, M. J. (1941). *Science*, **94**, 200.
KEARNEY, E. B., and SINGER, T. P. (1955). *Biochim. biophys. acta*, **17**, 596.
KREBS, H. A. (1933). *Z. physiol. Chem.*, **217**, 191.
KREBS, H. A. (1954). *In* Chemical Pathways of Metabolism, Greenberg D. M., Ed. Vol. I., p. 109. New York: Academic Press.
KREBS, H. A. (1956). *Dtsch. med. Wschr.*, **81**, 4.
KREBS, H. A., and KORNBERG, H. L. (1956). *Ergebn. Physiol.*, in press.
LARDY, H. A., and WELLMAN, H. (1952). *J. biol. Chem.*, **195**, 215.
LARDY, H. A., WIEBELHAUS, V. D., and MANN, K. M. (1950). *J. biol. Chem.*, **187**, 325.
LEPAGE, G. A. (1950). *Cancer Research*, **10**, 77.
LOHMANN, K., and MEYERHOF, O. (1934). *Biochem. Z.*, **273**, 60.
LYNEN, F. (1941). *Liebigs Annalen*, **546**, 120.
MARTIUS, C. (1956). Conférences et Rapports, III Int. Congr. Biochem., p. 1.
MENDEL, B. (1929). *Klin. Wschr.*, **8**, 169.
MEYERHOF, O., and KIESSLING, W. (1933). *Biochem. Z.*, **264**, 40.
NEEDHAM, D. M., SIMINOVITCH, L., and RAPKINE, S. M. (1951). *Biochem. J.*, **49**, 113.
POTTER, R. van, and REIF, A. E. (1952). *J. biol. Chem.*, **194**, 287.
RECKNAGEL, R. O., and POTTER, V. R. (1951). *J. biol. Chem.*, **191**, 263.
RUDNEY, H. (1949). *Arch. Biochem.*, **23**, 67.
WEIL-MALHERBE, H., and BONE, A. D. (1951). *Biochem. J.*, **49**, 339.

DISCUSSION

Brachet: Prof. Krebs, would you comment on the possible rôle of cell structure in this regulatory activity of the cell?

Krebs: A recent number of *Nature* contains a letter by Prof. Peters on this point (1956, *Nature, Lond.*, **177**, 426). He discusses the possible rôle of what he calls the cytoskeleton of the cell, a hypothetical network of structures which keeps enzymes and substrates in their places. He suggests that hormones may change the nature of this cytoskeleton and that hormones may not act directly on a specific enzyme; that perhaps one hormone can act on a number of enzymes at the same time, by making substances accessible. This is perhaps a useful working hypothesis, but it is difficult to visualize how one can test this idea by experiments. I would certainly agree that the structure is a very important point.

Alexander: I would like to ask a very similar question, but phrased in a slightly different way. You have told us that in the whole chain of enzyme reactions there are certain pacemakers. Now there must be a

certain step which is rate-controlling for the pacemaking reaction. In a heterogeneous system, as in the cell, there must be for each individual enzymatic stage a number of steps which are physicochemically distinct, such as diffusion of substrate to the enzyme and diffusion of the product away from the enzyme in addition to the actual chemical reaction occurring on the enzyme. Any one of these steps could determine the overall rate of the chemical reaction. Could you tell us whether the rate-controlling step of the individual enzyme reactions in the cell is likely to be the actual chemical reaction on the enzyme or a diffusion process?

Krebs: I don't think that one can make a general statement about it. What one can tackle experimentally is the identification of pacemaker steps, by determining the steady-state level of the intermediate metabolites, especially of the substrate of the pacemaker. If a substance reacts at a variable rate, its steady-state concentration must vary, because it will be produced at the same rate but removed at a different rate. A number of people have started to determine the steady-state level of intermediate metabolites, such as DPN, or reduced DPN, organic phosphates. This may lead to the identification of a pacemaker. But what changes the rate of such a reaction is a different matter. Before this can be answered the mechanism of the pacemaker reaction must be known. We just don't know enough about such mechanisms to say what rôle diffusion might play.

Dale: Do you consider it useful to do model experiments which may show effects on surfaces which bind enzymes and perhaps substrates at the same time, e.g. to imitate perhaps internal cell boundaries or cell surfaces with model experiments, such as burning of glucose on charcoal?

Krebs: In general I would think that the scope of model experiments is nowadays limited. The earlier models, like the oxidation of sugars on charcoal, would be largely irrelevant. We should study the real thing for preference.

Dale: It must be extremely difficult to determine the steady state in the cell for one of these recommended steps, because of their very small amounts present at a given time.

Krebs: It is indeed a great problem but it is being tackled and is being successfully solved in some cases. With paper chromatography and isotope techniques it is possible to determine quantitatively metabolites in very small amounts.

Dale: With regard to radiation it is difficult; you have to irradiate practically at the same time, because the steady state may change as soon as radiation stops.

Krebs: One can stop reactions very quickly under most conditions, by liquid air and other means.

Haddow: Did I understand you to mention hexokinase in relation to the cell surface?

Krebs: Yes, there is a good deal of evidence showing that the entry of sugar into the cell is not by passive diffusion but an active process. We have recently carried out experiments on the true sugar content of a number of tissues, using chromatographic separation, and they confirm

the older experiments that the sugar content of most tissues is indeed extremely low, of the order of 10–15 mg. per cent in the case of muscle, brain and testicle. Sugar was taken to diffuse very readily into cells, but this low concentration indicates that there is some barrier at the cell surface and that transport into the cell is something "active". Perhaps phosphorylated sugar only is transported into the cell, in which case the hexokinase would be required to be in the surface of the cell; but this is merely an idea. The point I made is that the hexokinase reaction itself or a step preceding it, dealing with the entry of sugar into the cell, is the reaction which initiates the degradation of sugar in the cell.

Haddow: We have become very interested in the properties of the cell surface in malignant cells and homologous normal cells. There are charge differences.

Zamecnik: Prof. Krebs, would you comment on the reactions leading toward synthesis of nucleic acid as possible rate-limiting steps? We are just beginning to get a more complete description of the series of reactions leading to nucleic acid synthesis, and I wonder if they may not be as likely a site as the steady-state conditions involved in glycolysis and oxidative phosphorylation.

Krebs: There must, of course, be rate-limiting reactions for many processes. What I have discussed in detail, because we have information on them, are the energy-giving reactions. The synthesis of nucleic acids is one of the processes dependent on energy supply. But its rate certainly does not depend merely on how much ATP is available. There must be some other mechanisms which control it. I have no idea of what these might be. I certainly agree that every complex synthesis must have a component which determines its rate. I should emphasize again the principle that there may be some reactions which are of less interest, if you study the effect of extraneous agents, than others which are more relevant because they determine the overall rate.

Lajtha: In connection with that point we have found that uracil deoxyriboside is readily methylated and gets into DNA thymine with relatively low concentration of inorganic phosphate in the medium; but cytosine deoxyriboside needs a high concentration of inorganic phosphate in the medium to do the same. Could that inorganic phosphate be already an energy-giving substance?

Krebs: I don't visualize any direct connection, but I must make it clear that the ideas which I have put forward cannot explain everything, they are merely meant to give some guidance in experimentation. They do not throw light on why you need a high concentration of phosphate in one case and not in another.

Cohn: In connection with the question on the biosynthesis of the nucleic acids, it is quite clear now that they begin with rather small molecules, e.g. with hexoses, trioses and dioses to build the ribose and deoxyribose moieties, formate to fill in the place in the purine ring as well as to add the methyl group, glycine, etc. So it seems to me that any influence upon such steps as these must be reflected in the amounts of substrates available for the build-up of nucleic acids, and thereby have some effect, however remote.

THE INFLUENCE OF OXYGEN ON RADIATION EFFECTS

H. Laser*

Molteno Institute, University of Cambridge

It is well established that the extent to which a variety of cells may be damaged by ionizing radiation is greater if the irradiation takes place in the presence of oxygen than if it takes place under anaerobic conditions. This applies equally to plant, insect and mammalian tissues (Barron, 1952; Hollaender, 1952; Hollaender, Baker and Anderson, 1951; Hollaender, Stapleton and Martin, 1951; Gray, 1953; Gray *et al.*, 1953), and the mechanism of this so-called "oxygen effect", which is not yet fully understood, may also hold the key to the mode of action of ionizing radiation in general.

Broadly speaking, two disciplines, with their sometimes widely varying train of thought and approach, have attempted to explain the oxygen effect, those of the physicochemist and the biochemist.

The former maintains that oxygen acts *per se*, e.g., that its mere presence modifies the nature of the chemical intermediates formed along the tracks of ionizing particles, thereby apparently producing greater though not well defined damage to the cells; or it thinks in terms of strongly oxidizing radicals, such as HO_2, and of H_2O_2, which are only formed in presence of dissolved oxygen, as causing increased radiosensitivity.

The difficulty in accepting a purely physicochemical explanation lies, at least to my mind, in the fact that it creates an unwarranted barrier between chemical and biochemical causes. It fails to take into account the fact that the state of the affected "entity" in the cell may determine its response to irradiation. It furthermore assumes that oxidizing radicals or

* Member of the Scientific Staff of the Medical Research Council.

agents affect biological systems which are in any event in a predominantly oxidized steady-state equilibrium; or that they oxidize reduced substances, e.g. SH groups (Barron, 1952) which are normally *reversibly* oxidized and reduced. Although such groups may be oxidized by radiation-produced radicals, they will only be eliminated from participation in further metabolic reactions if the oxidation thus produced is irreversible. That that is not generally the case follows, e.g., from the fact that anaerobic fermentation of yeast is not affected by fairly large X-ray doses which, however, strongly inhibit the ability to reproduce. Similarly the work of Pirie, van Heyningen and Boag (1953) and van Heyningen, Pirie and Boag (1954) on cataract induction by X-rays has shown that the glutathione content, total protein-SH and the activity of glutathione reductase in the lens were unaffected during the first 20 hours after irradiation, while the activity of a number of SH-enzymes begins to fall together with the onset of clinical cataract only weeks after irradiation. The authors believe that these changes may not constitute primary effects.

The biochemical approach which I propose to adopt with regard to the oxygen effect visualizes that:

(1) only in the presence of oxygen is the enzymic equilibrium within the cells such that they are most severely affected by irradiation products of water, the primary step being reduction by hydrogen atoms followed by secondary oxidation through either molecular oxygen or oxidizing radicals, which, however, leads to abnormal, irreversibly oxidized products;

(2) the oxygen effect, as expressed, e.g., by inhibition of bacterial growth, occurs only if the cells maintain a certain minimal metabolic activity and possess the entire enzymic make-up (or at least its precursors) necessary for subsequent growth and protein synthesis, during the actual irradiation;

(3) many substances and cell constituents which protect the cell from irradiation do not do so effectively in the presence of oxygen.

Experimental data in support of these three propositions will be given.

It has previously been shown (Laser, 1954) with the bacterium *Sarcina lutea*, that the oxygen effect on the rate of reproduction could be largely abolished by doses up to 26 kr if cell respiration was inhibited by respiratory poisons during the irradiation. Thus, after removal of the poison, the cells behaved as if they had been irradiated in nitrogen, when judged by the degree of growth inhibition. The effective inhibitors were carbon monoxide, potassium cyanide, hydroxylamine and sodium azide. Urethane did not diminish the oxygen effect (Fig. 1). The mode of action of the effective poisons in their rôle as respiratory inhibitors is known (Keilin, 1933; Keilin and Hartree, 1939; Keilin and Slater, 1953). They all block hydrogen transfer through the respiratory enzymic system by combining with cytochrome a_3 and stabilizing the remaining respiratory enzymic chain in the reduced form. Taking this mode of action as a guide in advancing a possible explanation for the oxygen effect in irradiation, it is suggested, at least for this bacterium, that the enhancement of irradiation damage (1) involves the enzymic respiratory mechanism, (2) requires at least part of the enzymic respiratory chain to be in the oxidized form during irradiation. This supports the view that the impedance has been caused by a reducing agent. These results and their interpretation have recently been corroborated by Tahmisian and Devine (1955) who have shown that grasshopper eggs which show a certain regression, "negative growth", when irradiated in air are less affected if the nitrogen of the air is replaced by carbon monoxide, i.e., in 20 per cent O_2/CO. The protection by carbon monoxide is light-sensitive, being effective only in the dark. That X-ray induced inactivation of biological material is brought about by reduction has also been shown for two different strains of bacteriophage, by Ebert and Alper (1954) and by Bachofer and Pottinger (1954). It should, however, be added that the view that reducing agents are generally responsible for irradiation damage has been contradicted. Thus, Forssberg's (1947) claim, that catalase in aqueous solution is inactivated by means of reducing hydrogen atoms produced by X-irradiation,

has not been supported by Sutton (1952), who found that hydrogen atoms produced by means other than irradiation did not inactivate catalase, and that irradiation in presence of oxygen was more damaging than in an atmosphere of

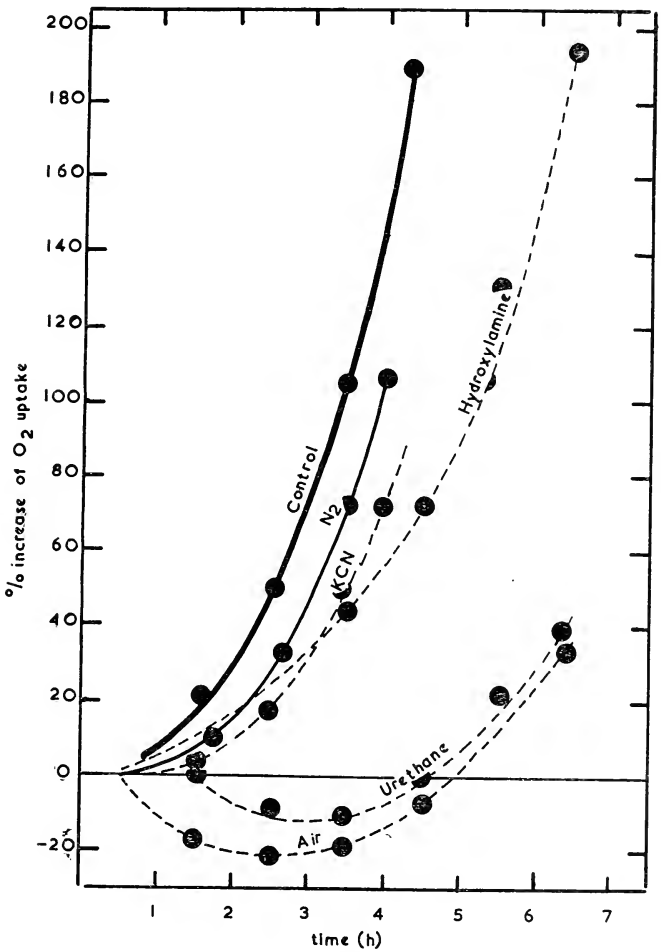


FIG. 1. Percentage increase in oxygen uptake (= growth) of *Sarcina lutea* during irradiation with 26 kr X-rays, in nitrogen and in air ± potassium cyanide, hydroxylamine and urethane.

hydrogen; nor has it been confirmed by Dale and Russell (1956).

Furthermore, the involvement of haematin compounds follows from experiments with *Escherichia coli* (Fig. 2), in which the influence of irradiation on respiration and on aerobic acid-production of washed non-growing cells has been measured.

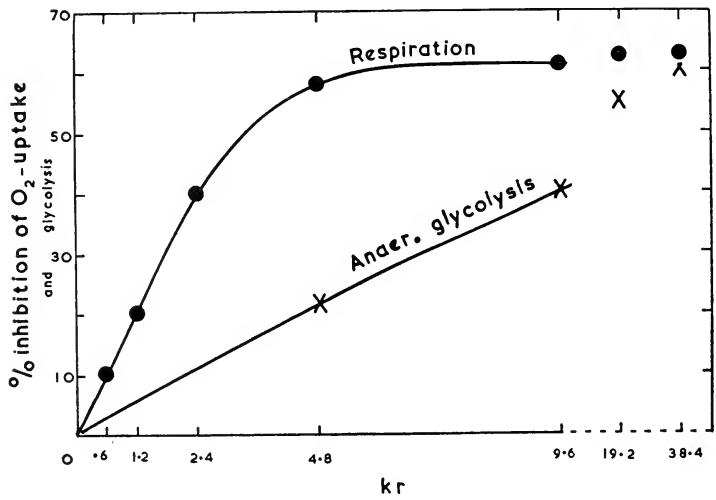


FIG. 2. Percentage inhibition of respiration and anaerobic glycolysis of washed suspensions of *Esch. coli* irradiated in air with increasing dose of X-rays.

The rate of oxygen uptake in the presence of glucose was progressively inhibited with increasing doses of X-rays, up to a maximum of approximately 65 per cent at 5,000 r. The remaining 35–40 per cent respiration was found to be cyanide-insensitive and could not be further reduced by increasing the X-ray dose sixfold, although reproduction was progressively more inhibited. Inhibition of anaerobic acid production (after aerobic irradiation) follows a different course from that of respiration but reaches the same plateau at about 30 kr. Here, again, the remaining anaerobic acid production was

unaffected by sodium azide which, with *Esch. coli*, is an inhibitor of anaerobic glycolysis.

It is conceivable that haematin compounds in the cell behave in the same way as haemoglobin and cytochrome *c*, which are first reduced by X-radiation and then partly reoxidized in a secondary reaction by molecular oxygen and by radicals to an unnatural, green pigment, to which extent they lose their oxygen-carrying and catalytic properties (Laser, 1955). The fact that, with the exception of *Esch. coli*, the aerobic and anaerobic metabolism of non-growing suspensions of a fairly large and representative number of bacteria and of yeast was not affected—even to the extent of uninhibited adaptive enzyme formation (Baron, Spiegelman and Quastler, 1952–53)—by X-ray doses which inhibit growth by more than 90 per cent may be taken to indicate that the complement of haematin compounds in the cell involved in these reactions is not a limiting factor but may be so in relation to induction of growth.

That the prerequisite for the oxygen effect is a certain state and/or metabolic activity of the cell during irradiation is demonstrated by the following two types of experiment:

(1) Spores of *Bacterium subtilis* were irradiated under varying conditions. They were then transferred to a growth-promoting medium in manometer flasks, and the rate of growth, i.e. the formation of vegetative forms and subsequent reproduction, was determined by measuring the increase in oxygen uptake with time, which under normal conditions follows a logarithmic course and is a true measure of the increase in the number of cells. *Fresh* spores, i.e., those harvested soon after sporulation, irradiated either dry (not dried) or suspended in phosphate buffer, showed about the same radiosensitivity as vegetative forms and an oxygen effect of the same order. However, if the spores, after thorough washing, had been freeze-dried prior to being irradiated, they were somewhat less radiosensitive and showed no oxygen effect (with doses up to 36 kr) when resuspended in buffer and irradiated in the liquid phase.

(2) The oxygen effect, which is very marked with fresh yeast (Fig. 3), was similarly abolished with yeast (*Candida utilis*) which had been starved of nitrogenous reserves ("low-nitrogen" yeast) by depriving it, in presence of oxygen and a carbon source (glucose), of added nitrogenous substrate for 1–2 days prior to irradiation (Fig. 4). Such a yeast has a very low (resting) metabolism. When it is brought into a nutrient

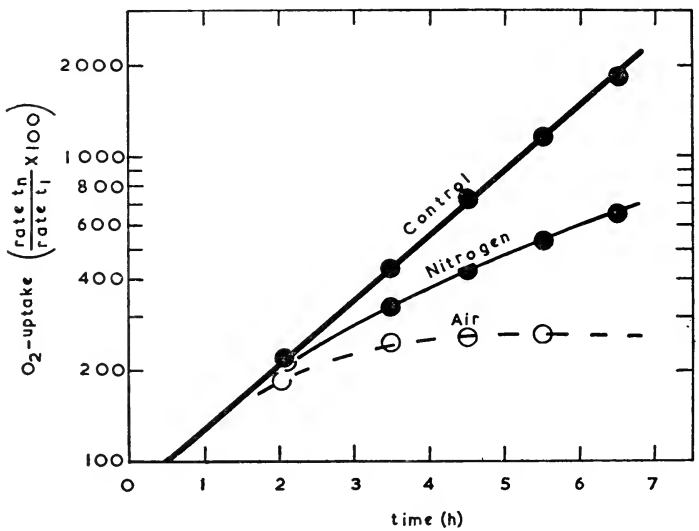


FIG. 3. Percentage increase in O_2 uptake (= growth) of yeast (*Candida utilis*) after irradiation in air and in nitrogen with 24 kr X-rays.

(nitrogenous) medium the oxygen uptake per cell increases before growth sets in. It is reasonable to assume that the nitrogen-depleted cell has to replenish its relevant enzymic make-up before starting to divide. As the depleted cell shows no oxygen effect, it follows that the sensitive structure was either not present or was in a state in which the presence of oxygen did not affect its radiosensitivity.

Lastly, I would like to discuss experiments dealing with protection from irradiation by means other than reducing agents, such as cysteine. Dale (1940, 1942) has already shown

that carboxypeptidase and the prosthetic group (alloxazine-adenine dinucleotide) of D-amino-acid oxidase could be largely protected not only by their specific substrates but also by a variety of substances which are in no way structurally related to these substrates. However, the problem of protection does not seem to have been examined in relation to the

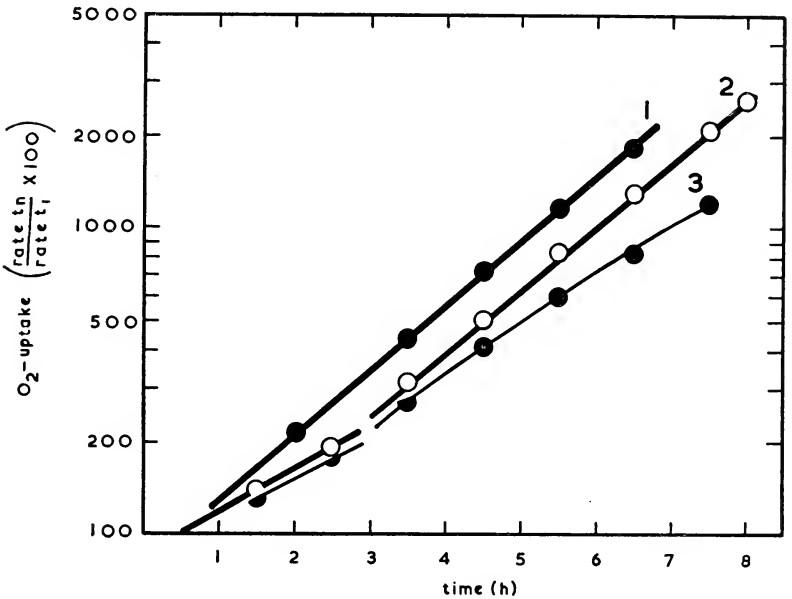


FIG. 4. Percentage increase in O_2 uptake (= growth) of untreated and "low-nitrogen" yeast (see text).
(1) unirradiated control;
(2) unirradiated "low-nitrogen" yeast;
(3) "low-nitrogen" yeast irradiated in air and in nitrogen (24 kr.)

oxygen effect, except by allowing bacteria to deplete their medium of dissolved oxygen by oxidation of added substrate, e.g. succinic acid, without renewing the used-up oxygen (Stapleton, Billen and Hollaender, 1952), a procedure which in effect does not differ from the removal of oxygen by reducing agents or by physical means. The enzyme used for the

experiments to be described was a flavoprotein, namely notatin (glucose oxidase) (Keilin and Hartree, 1948). By means of a manometric micro-method for determining the *initial* rates of glucose oxidation by small amounts of enzyme (< 1 μg.) inactivation of the enzyme of the order of 10–20 per cent could be reproducibly detected with accuracy. Table I, dealing with

Table I

Addition	*Notatin, 20 μg./ml. percentage inactivation by 38·4 kr in*	
	Air	N_2
—	15	95–100
Glucose	50–60	15– 20
Other sugars	15	15– 20
Albumin, 0·1 %	15	30
Glucose + albumin	45	—
Gelatin 0·1 %	—	70

the application of 38 kr X-rays reveals the, at first sight surprising, result of a reversal of the oxygen effect, the enzymic activity being practically completely inhibited by irradiation in nitrogen but only slightly (15 per cent) depressed if oxygen is present. However, the presence of glucose, the specific substrate, during irradiation in air brought about a strong inhibition (50 per cent) while the presence of glucose during irradiation in nitrogen protected, lessening the inactivation from 100 per cent to 15 per cent. Catalase, in catalytic amounts, did not alleviate the inhibition which glucose caused during irradiation in air, i.e., the enzyme had not been partially inactivated either by enzymically or radiochemically produced H_2O_2. The inactivation of notatin on irradiation in air was observed only in the presence of its specific substrate. A number of other sugars, which are not oxidized by the enzyme, caused no inactivation in air but exerted a high degree of protection during irradiation in nitrogen. Similarly, added protein (albumin), which greatly protected against irradiation in

nitrogen, decreased only slightly the inactivation which the addition of glucose induces in air.

It may be legitimate to conclude that in this particular case the enzyme seems most likely to be radiosensitive when it is in the form of a semiquinone (Kuhn and Wagner-Jauregg, 1934; Michaelis, Schubert and Smythe, 1936; Haas, 1937). These data offer further support to the more general conclusion that (1) the oxygen effect, as already pointed out above, is related to enzymic activity during the actual irradiation; (2) the points of attack within the cell or on an enzyme molecule, as well as the damaging agents, differ widely depending on the presence or absence of oxygen. The result will therefore depend on the relative rôle which the affected group plays in the economy of the cell.

REFERENCES

BACHOFER, C. S., and POTTINGER, M. A. (1954). *Science*, **119**, 378.

BARON, L. S., SPIEGELMAN, S., and QUASTLER, H. (1952–53). *J. gen. Physiol.*, **36**, 631.

BARRON, E. S. G. (1952). Symposium on Radiobiology, p. 216. New York: Wiley.

DALE, W. M. (1940). *Biochem. J.*, **34**, 1367.

DALE, W. M. (1942). *Biochem. J.*, **36**, 80.

DALE, W. M., and RUSSELL, C. (1956). *Biochem. J.*, **62**, 50.

EBERT, M., and ALPER, T. (1954). *Nature, Lond.*, **173**, 987.

FORSSBERG, A. (1947). *Nature, Lond.*, **159**, 308.

GRAY, L. H. (1953). *Brit. J. Radiol.*, **26**, 609.

GRAY, L. H., CONGER, A. D., EBERT, M., HORNSEY, S., and SCOTT, O. C. A. (1953). *Brit. J. Radiol.*, **26**, 638.

HAAS, E. (1937). *Biochem. Z.*, **290**, 291.

HEYNINGEN, R. VAN, PIRIE, A., and BOAG, J. W. (1954). *Biochem. J.*, **56**, 372.

HOLLAENDER, A. (1952). Symposium on Radiobiology, p. 285. New York: Wiley.

HOLLAENDER, A., BAKER, W. K., and ANDERSON, E. H. (1951). *Cold Spr. Harb. Symp. quant. Biol.*, **16**, 315.

HOLLAENDER, A., STAPLETON, G. E., and MARTIN, F. L. (1951). *Nature, Lond.*, **167**, 103.

KEILIN, D. (1933). *Ergebn. Enzymforsch.*, **2**, 239.

KEILIN, D., and HARTREE, E. F. (1939). *Proc. roy. Soc. B.*, **127**, 167.

KEILIN, D., and HARTREE, E. F. (1948). *Biochem. J.*, **42**, 221.

KEILIN, D., and SLATER, E. C. (1953). *Brit. med. Bull.*, **9**, 89.

KUHN, R., and WAGNER-JAUREGG, T. (1934). *Ber. dtsch. chem. Ges.*, **67**, 361.

LASER, H. (1954). *Nature, Lond.*, **174**, 753.
LASER, H. (1955). *Nature, Lond.*, **176**, 361.
MICHAELIS, L., SCHUBERT, M. P., and SMYTHE, C. V. (1936). *J. biol. Chem.*, **116**, 587.
PIRIE, A., HEYNINGEN, R. VAN, and BOAG, H. W. (1953). *Biochem. J.*, **54**, 682.
STAPLETON, G. E., BILLEN, D., and HOLLAENDER, A. (1952). *J. Bacteriol.*, **63**, 805.
SUTTON, H. C. (1952). Radiation Chemistry, Disc. Faraday Soc., no. 12, p. 281.
TAHMISIAN, T. N., and DEVINE, R. L. (1955). *Radiation Res.*, **3**, 182.

DISCUSSION

Alper: Mr. Paul Howard-Flanders and I have some results which are relevant to what Dr. Laser has been discussing. We took *Esch. coli* B in a starving condition and bubbled oxygen through them for a good long time, so that they should have exhausted most or a good deal of their endogenous substrate, in order to see what the radiosensitivity under nitrogen would be. At first it seemed as if the nitrogen survival curve was quite different if one had treated the bacteria in this way; but a more rigorous examination of this phenomenon showed that it was, in fact, due to the circumstance that the sensitivity of *Esch. coli* B is affected by extremely low oxygen concentrations. This means, of course, that in order to distinguish very clearly between the survival curves you get under oxygen or under complete anoxia, you have to have extremely rigorous conditions of oxygen exclusion. These survival curves were all done with very dilute suspensions of bacteria, and with suspensions in which the gas mixture was bubbling through continuously, so that one could feel fairly confident at least about the oxygen tension in the fluid surrounding the cells. Now there are several points relevant to Dr. Laser's argument about the necessity for the cells to be metabolizing in order to show the oxygen effect. Longmuir (1954, *Biochem. J.*, **57**, 81) gives a figure which is analogous to the Michaelis constant, the oxygen concentration at which the bacteria are respiring at half their normal rate. This concentration is $2{\cdot}2 \times 10^{-8}$ M oxygen, and on this basis they would be respiring at 95 per cent of their normal rate at something like 4×10^{-7} M; the region within which they are reaching full oxygen radiosensitivity, however, is something of the order of 10–20 μM. We have also done survival curves with these various gas mixtures in the absence and in the presence of substrate. When we compared the curve which represents the points in the absence of substrate, with the curve obtained when succinate was added, there was no difference for the nitrogen point, for complete oxygenation, or for 0·02 per cent oxygen. There was, in fact, only one point where the succinate apparently made a slight difference, and since it was only at the one point I think this will certainly need verification. Apart from this experiment the succinate made no difference at all to the survival curves.

Gray: It is clear that the position of the bend in now very much in need of being looked at again.

Previous studies with X-rays by Read, who observed root growth in *Vicia faba*, by Giles, who observed chromosome structural damage in *Tradescantia* microspores, and by Baker and others, who observed chromosome structural damage in *Drosophila* sperm, all showed this bend to occur when the partial pressure of oxygen in the gas phase was about the same as in air, corresponding to a molarity of oxygen dissolved in water at room temperature of around 250 μM/litre. This contrasts with the situation indicated by Miss Alper for bacteria, where the bend occurs at about 50 μM/litre dissolved oxygen. The contrast is the more striking if expressed in terms of the molarities of dissolved oxygen which confer half the difference between the anaerobic and the fully aerobic sensitivities; these are around 7 μM/litre for bacteria and 120 μM/litre for the other materials. Each of the other materials referred to has been irradiated as tissues, not as single cells, and the observed relation between sensitivity and oxygen tension must in each case have been influenced in some degree by the gradient of oxygen tension throughout the tissue arising from cellular respiration. The point has been considered by the authors concerned and believed not to be one of major importance, though definite experimental evidence is lacking. This factor cannot have been operative, however, in the experiments of Conger, who irradiated ascites tumour cells in a suspension in equilibrium with known gas mixtures and obtained a relation essentially the same as that which had been obtained for the roots, microspores, and *Drosophila* sperm. The procedure used in Conger's experiments was that the fluid in which the cells were suspended was stirred vigorously by a jet of gas of known composition. The molarity of the dissolved oxygen was not measured. In view of Miss Alper's results it becomes a matter of importance to check Conger's observations. It will be recalled that, using the same experimental procedure, Conger found only a very slight influence of oxygen on the sensitivity of ascites tumour cells to neutron radiation which, by analogy with the relatively slight influence of oxygen tension on the chemical reactions induced in aqueous solutions by high ion density radiations, lent support to a chemical—as against a biochemical—type of explanation. In other cases the evidence is less clearcut. Dr. Laser referred to the influence of carbon monoxide on the X-ray sensitivity of his bacteria. It will be recalled that Sachs found that carbon monoxide at four atmospheres pressure in the presence of air increased damage to *Tradescantia* microspores. I found similar effects in roots but only when the carbon monoxide pressure was increased to about 20 atmospheres. Moreover, I found a much smaller influence of carbon monoxide on growth inhibition induced by exposure to neutrons. While these facts would be most readily accounted for in terms of radiation chemistry, the fact that the enhanced radiosensitivity was a function of the period of carbon monoxide pretreatment, which was only fully effective if applied for 30 minutes prior to as well as during irradiation, suggests that metabolic factors were involved.

Alper: There is one point I would like to make. I don't know whether our results at these extremely low oxygen concentrations are good enough to be quite sure that we failed to demonstrate the effect I mean. If you take the view that it is only after the respiratory chain is fully oxidized that you get an oxygen effect, you should in fact have a little tail to this curve. In the experiments on bacteria carried out by Dr. Hollaender's group, quite a big tail is shown in the oxygen effect curve. They worked with a closed system and a dense suspension of bacteria so that they were able to use up the oxygen and did in fact do so; if the results are plotted out in this way you do get quite a tail to that curve. We get the oxygen at these very low tensions by producing it with an electrolysis cell and running the cell at currents from 10 mA upwards, so that we can get something of the order of 0·01 per cent oxygen or less. You get a very slight but real increase at that concentration, and increasing sensitivity as oxygen concentration increases; there is some scatter in the points, but the results show this clearly.

Stapleton: Dr. Billen in our laboratory followed the respiration of several strains of irradiated *Esch. coli*. He measured the consumption of oxygen by these irradiated cells, and compared this oxygen uptake with that of non-irradiated cells, using glucose as substrate. He found that the control cells, of course, consumed oxygen at a constant rate with time of incubation. Irradiated cells, on the other hand, although the final population as measured by ability to reproduce themselves was something like one viable cell in 10^5, consumed oxygen at approximately the control rate for a period of something like 40–50 minutes. Then there was a reasonably sharp break in the curve followed by a slow steady decay of the respiratory ability of the cells. I think what this curve means is that all the cells are viable during this period of time. The break may represent a change or a beginning of death of the population. Billen did some further experiments to see if some correlation could be made with ATP synthesis in the cells and found that under the same conditions the irradiated cells can synthesize ATP at approximately the same rate as control cells. At about the same time that this break in the oxygen uptake occurs, although the cells were making ATP at approximately the normal rate, something like 80 per cent of ATP synthesized by the cell was found outside rather than inside the cell. This again could mean that the population is changing with respect to viability.

Latarjet: We have material that could be of use to Dr. Laser. Dr. Beljanski has treated several strains of *Esch. coli* with large amounts of streptomycin on minimal medium, and finally isolated some stable mutant strains which are unable to synthesize the porphyrin ring. They are haemin minus and if you grow them on small amounts of peptone you get bacteria which have none or at most 1/500th the total porphyrins of the normal strain. They have no catalase, no peroxidase, no cytochrome, or at most very little. We were interested, not in the oxygen effect, but in photorestoration. (Incidentally, these strains are as photorestorable as the wild ones.) But, hearing Dr. Laser, I wonder if such

a material would not be of help in work with ionizing radiations, to see what the oxygen effect would be in these strains.

Laser: It would certainly be of great help to me and I would be grateful to obtain such a strain of *Esch. coli.* With regard to Dr. Stapleton's statement that the respiration curve shows a sharp break after about 40 minutes, which amounts practically to a cessation of respiration, I have never had that experience in extensive studies with a great variety of bacteria.

THE INFLUENCE OF CHEMICAL PRE- AND POSTTREATMENTS ON RADIOSENSITIVITY OF BACTERIA, AND THEIR SIGNIFICANCE FOR HIGHER ORGANISMS*

ALEXANDER HOLLAENDER AND GEORGE E. STAPLETON
Biology Division, Oak Ridge National Laboratory, Tennessee

PROTECTION against ionizing radiation by chemicals can be discussed on the basis of model experiments with polymers or on experience with living materials. In some ways, experiments conducted with chemicals and living cells fit into a pattern related to model tests; however, for the purpose of this discussion, model experiments are not sufficient since they do not give information on metabolizing systems. The most extensive work in the field of model experiments has been done by Alexander, Charlesby, and Ross (1954). A large number of compounds that protect readily against radiation in the polymers are not necessarily effective on biological materials, but they could serve as a guide to practical applications. Alexander and co-workers (1955) have shown a close parallelism between the protective ability of many compounds for polymers and the survival of irradiated mice. The number of compounds that actually protect mice to a highly significant degree, and with a minimum of detrimental effects, is relatively small. Our interpretation was that these compounds compete for the oxygen present in the suspension and/or for radiation-produced radicals and peroxides. Alexander and co-workers (1955) believe that these compounds act chiefly by competing for HO_2 radicals. On the basis of present knowledge, it is very difficult to distinguish between these two mechanisms since

* Work performed under contract W-7405-eng-26 for the Atomic Energy Commission in the Biology Division, Oak Ridge National Laboratory.

oxygen removal is not always so efficient as chemical protection. As a matter of fact, the dose-reduction factor (DRF) for *Escherichia coli* will go as high as 12 under the best conditions for cysteamine protection (Hollaender and Doudney, 1955) in contrast to 3 for oxygen removal by replacement (Hollaender, Stapleton, and Martin, 1951). The difference between these dose-reduction factors probably is based on the premise that replacement of oxygen by an inert gas (Doudney and Hollaender, 1956)—for instance, nitrogen—might not completely remove the oxygen, whereas sodium hydrosulphite (Burnett *et al.*, 1951) or cysteamine, since both are highly water-soluble, can enter the cell and remove the oxygen, especially when it is in loose association with certain compounds or is adsorbed on the surface of particular cell structures. Several investigators have shown that cysteamine can associate with nucleic acids and other compounds (Kluyskens, 1953).

Two compounds that appear to be of greater importance, because of their striking protective ability, are β-mercaptoethylamine (MEA) and *S*-β-aminoethyl*iso*thiouronium·Br·HBr (AET). The former is apparently the most successful compound for *Esch. coli*, and the latter for mice.

Cysteamine (MEA), first reported by Bacq and co-workers (1951) to be protective for mice and since found to be an effective chemical protective agent by workers in many other laboratories, is an easily oxidized compound, has to be stored under nitrogen, and has other characteristics that make it somewhat difficult to handle. In freshly prepared water suspension, there is increasing protection for *Esch. coli* B/r (Fig.1) with increasing concentration until a plateau is reached at 0·02 M. Phosphate buffer interferes somewhat with the protection, and the protective ability is dependent on the nutritional factors supplied after irradiation. The effect of cysteamine on *Esch. coli* may be summarized as follows (Fig. 2):

1. It protects in a concentration of 0·02 M and has a dose-reduction factor of 12 if broth-grown cells are incubated in a complete medium.

2. Cells grown in inorganic salts and glucose do not require a complete medium for highest protection.

3. Significant protection is still obtained if cysteamine is washed off the cells immediately before irradiation, probably a large part of the MEA is absorbed by the cell.

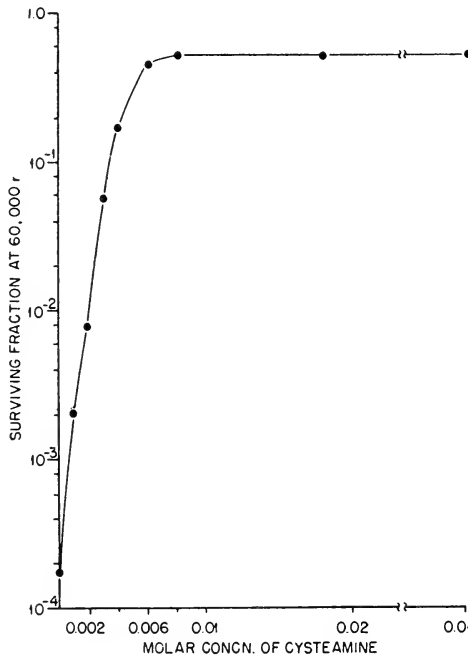


FIG. 1. Effect of cysteamine on the survival of *Esch. coli* B/r during exposure to 60 kr of 250-kv X-rays.

4. Limiting concentrations of cysteamine do not change the shape of the survival curves; however, reduced survival is obtained at each dose level.

5. The protection is somewhat strain-dependent; a careful technique must be used for the highest expression of protective ability. Some points can easily be obscured by changing some conditions of experimentation.

The excellent protective ability of cysteamine for *Esch. coli* and its limitations in animal experiments have stimulated David G. Doherty (Doherty and Burnett, 1955; Doherty and Shapira, 1956) of our laboratory to prepare more than 100 derivatives of this compound. Time is too short for a review

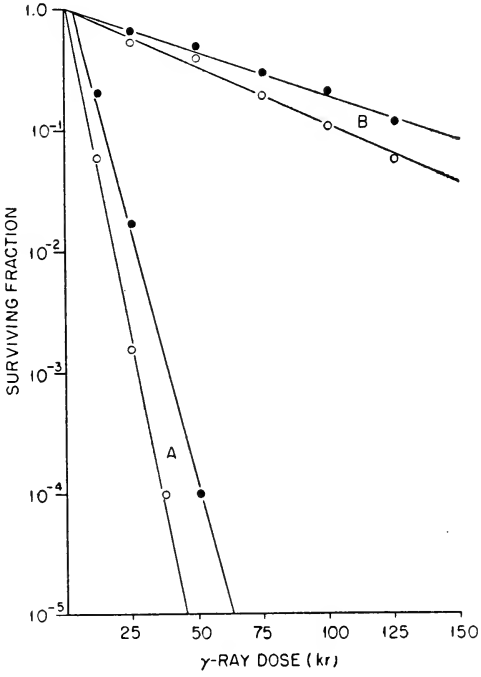


FIG. 2. Gamma-ray sensitivity of *Esch. coli* B/r in the presence and absence of cysteamine. ●, Yeast extract; O, basal medium; A, cells irradiated in H_2O; B, cells irradiated in H_2O + 0·02 M cysteamine.

of the functions of all these compounds; therefore, only the results are summarized below:

1. The protective ability disappears when the number of carbons in the basic structure of AET is increased in excess of 3 (Fig. 3).

2. Different groups may be added to the sulphur, provided that their form is such that they do not unbalance the molecule.

3. The NH_2 group must be left free; otherwise, the toxicity increases or the protective ability is drastically reduced.

4. The compound (AET) is stable at pH 3 and can be hydrolysed only at pH's in excess of 8 with amino alkyl mercaptans and dicyandiamide as the breakdown products.

5. Compounds found to be protective give a positive –SH test at pH 7·5. Doherty's explanation for this is that AET exists in multiple forms, and equilibria among these forms can be changed by modifying the pH. Some of the different stages that AET goes through are shown in Fig. 3.

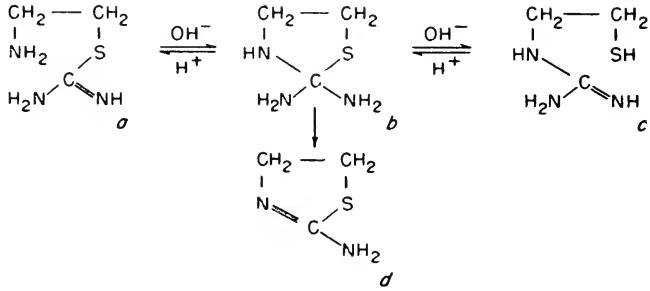


FIG. 3. Multiple equilibrium structures of AET.

6. All compounds that are active in protecting mice appear to be able to form a *b* type ring structure (Fig. 3) in solution at a neutral pH similar to a thiazoline ring.

7. A solution of AET appears to be a mixture of different types of isomers, whose forms are very similar to coenzyme A (Bashford and Huennekins, 1955) and glutathione (Calvin, 1954), which are known to exist in several isomeric forms.

Unfortunately, AET is not one of the best compounds for protecting *Esch. coli* but it is considerably more successful than cysteamine in protecting mice. It almost doubles the LD_{50} and is now used routinely in our laboratory for protection studies with mice.

From experiments on bacterial cells, we are just beginning

to understand some of the relationships of postirradiation (recovery) systems on the cell level. Some of the details of experimentation and results of these experiments belong in this discussion. Two points of view can be taken immediately: (1) the recovery system may prevent the disruption of key

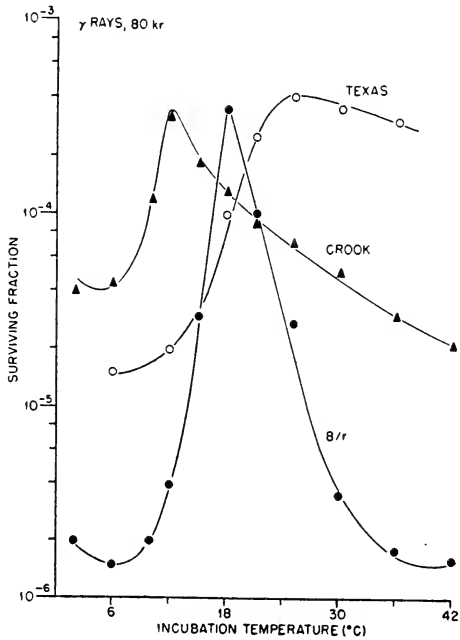


FIG. 4. Survival of three strains of *Esch. coli* at 80 kr at various temperatures. ▲, Crooks; ●, B/r; O, Texas. (Stapleton, Billen and Hollaender, 1953.)

biological material or (2) it may allow the cell to replace damaged material by synthesis. Both types of recovery may be demonstrated.

1. Reduced postirradiation incubation temperature increases the survival of several strains of *Esch. coli*. The B/r strain shows a true optimum for survival (Fig. 4) at 18° C after X- or gamma-rays (Stapleton, Billen, and Hollaender, 1953).

2. The effect is dose-independent, a true dose-reduction phenomenon (Fig. 5).

3. The effect is related to inactivation by free radicals. Dose-reduction factor is reduced for oxygen-free suspensions.

4. The survival of irradiated cells at any temperature may

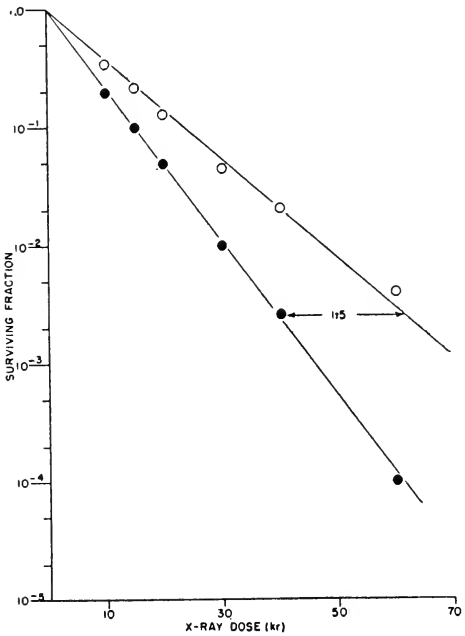


FIG. 5. Recovery at various X-ray doses (aerobic B/r 20-hour culture cells irradiated). O, Holding at 18° C; ●, holding at 37° C. (Stapleton, Billen and Hollaender, 1953.)

be related to the relative rates of synthetic and radiation-induced degradative processes.

An effect of the medium in which the cells are incubated is displayed at all the temperatures studied (Stapleton, Sbarra, and Hollaender, 1955) (Fig. 6). The pertinent results can be summarized as follows:

1. Additivity can be shown between the temperature and

medium effect. This finding illustrates that both points of view may actually be correct.

2. Extracts of natural materials (e.g., beef, yeast, or other tissue extracts) yield higher survival than that obtained on a

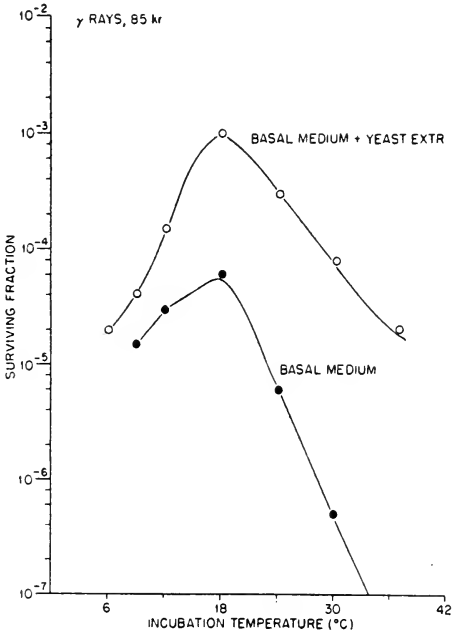


FIG. 6. Comparison of viability on basal medium and basal medium plus yeast extract as a function of postirradiation incubation temperature. (Stapleton, Sbarra and Hollaender, 1955.)

simple inorganic salts-glucose (Fig. 7) medium, although the latter medium is not limiting for normal (non-irradiated) cells.

3. Attempts to isolate the required materials indicated a multiple requirement.

4. A complex, chemically defined plating medium (Table I) will, to a high degree, substitute for the natural materials.

Deletion experiments showed the minimal (Table II) organic requirements to be guanine, uracil, and glutamic or aspartic acids, precursors for ribonucleic acid and/or protein synthesis.

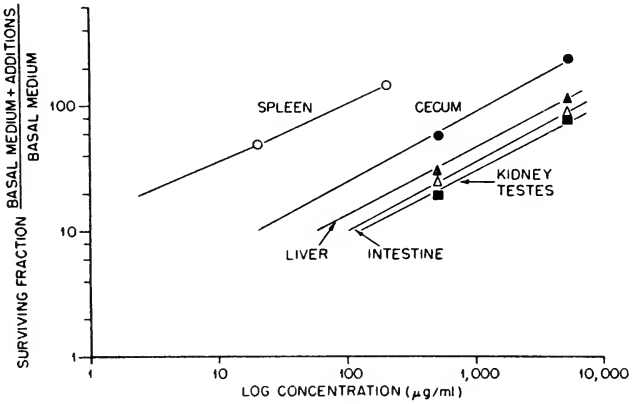


FIG. 7. Relative activity of rabbit tissue homogenates; incubation at 37° C. (Stapleton, Sbarra and Hollaender, 1955.)

It seemed of the utmost importance to investigate the effect of preirradiation growth conditions on the survival of cells after irradiation, as well as their response to added

Table I

COMPLETE SYNTHETIC MEDIUM

Components		*g./l. of medium*
Salts A	K_2HPO_4	0·5
	KH_2PO_4	0·5
Salts B	$MgSO_4$	0·1
	NaCl	0·01
	$FeSO_4$	0·01
	$MnSO_4$	0·01
Glucose		10

Plus or minus vitamins, amino acids, purines, and pyrimidines.

nutritional factors after exposure. Cells were grown in a variety of media including: (1) basal medium—the inorganic

salts-glucose medium, (2) basal medium fortified with amino acids, (3) basal medium fortified with purines and pyrimidines, (4) a combination of these, and (5) nutrient broth.

Table II

CHEMICALLY DEFINED RECOVERY MEDIUM

Components	*Amount*
Glutamine	150 μg.
Uracil	30 μg.
Guanine	30 μg.
Salts A	1 ml.
Salts B	1 ml.
Agar	3·4 g.
Glucose	2·0 g.
Distilled H_2O	200 ml.
pH 6·8	

After irradiation, cells grown on the various media were plated on the media described in Table III, and the following results were obtained:

Table III

EFFECT OF CULTURE MEDIUM ON RADIATION SENSITIVITY

	Surviving fraction of cells, after 85 kr of gamma-rays, plated at 37° C on:				
Culture medium	*Basal medium*	*Basal medium + guanine + uracil*	*Basal medium + caseamino acids*	*Complete synthetic medium*	*Basal medium + 20 mg. of yeast extr.*
Basal	$2·2\times10^{-6}$	$3·1\times10^{-6}$	$4·0\times10^{-6}$	$2·2\times10^{-5}$	$1·3\times10^{-5}$
Basal + guanine + uracil	$1·0\times10^{-7}$	$1·0\times10^{-6}$	$8·0\times10^{-7}$	$3·0\times10^{-6}$	$2·6\times10^{-6}$
Basal + caseamino acids	$2·3+10^{-7}$	$2·9+10^{-6}$	$2·3\times10^{-5}$	$5·0\times10^{-5}$	$6·0\times10^{-5}$
Nutrient broth	$3·0\times10^{-8}$	$5·0\times10^{-8}$	$8·0\times10^{-7}$	$2·7\times10^{-6}$	$3·5\times10^{-6}$

1. Cells harvested from basal medium did not show a striking response to added nutrilites. The survival as a function of dose indicated that they did not require the nutrilites since essentially similar survival was obtained on basal

medium as was obtained on fortified media by cells harvested from nutrient broth.

2. Cells harvested from basal media to which amino acids or purines and pyrimidines were added showed requirements for these materials after irradiation. Amino acids were most effective.

3. Cells harvested from nutrient broth showed the most clear-cut dependence of survival on the supplemented medium following irradiation.

Data on the effect of growth conditions prior to irradiation on the subsequent postirradiation requirements suggested that a sizeable part of the process called "recovery" is related to an adaptive process, and probably involves new enzyme synthesis. Cells that have been grown in a simple medium prior to irradiation might be expected to have a different complement of enzymes from those grown in a nitrogen-rich medium such as beef extract. The former cells have had to synthesize not only all metabolic precursors and intermediates from carbon fragments and ammonia but, probably, also the enzymes necessary for these synthetic reactions. The latter cells have had numerous intermediates supplied to them continuously during growth and, therefore, have probably been able to by-pass many synthetic reactions. They are essentially undeveloped or deficient cells. Non-irradiated cells, if transferred from a rich medium to the basal medium can adapt readily to the simple medium; but, interestingly, irradiation appears to interfere with such an adaptation. According to these studies it would seem that under optimal conditions—(1) reduced incubation temperature and (2) the presence in the postirradiation medium of precursors for ribonucleic acid and protein synthesis—a reasonably large fraction of the irradiated population appears to be able to perform the required syntheses.

Short-term incubation of irradiated cells with extracts of natural materials (15–30 minutes at 37° C) prior to plating, results in loss of dependence of the cells on the plating medium. Quantitatively similar survival is obtained on both

media. This incubation period is not sufficient to allow cell division to occur. Apparently, some nutritional factor is present in these extracts which either partially reverses the potential damage brought about by X- or gamma-rays or aids in the adaptive process involved. These two suggested mechanisms may actually be identical in the bacterial system.

The synthesis of ribonucleic and deoxyribonucleic acids, and of proteins, was followed in non-irradiated and in irradiated cells under the best and worst conditions for recovery,

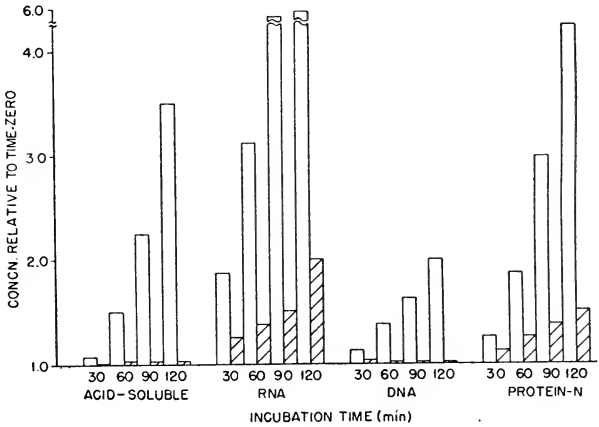


FIG. 8. Relative rates of synthesis of nucleic acids and protein by irradiated (▨) and non-irradiated (□) *Esch. coli* B/r on basal medium at 37° C.

i.e., incubation at 18° C in a complete medium and at 37° C in basal medium, respectively (Fig. 8) (Stapleton and Woodbury, 1955). Similar aliquots of cell suspension were inoculated into the two media at the two temperatures; and after various incubation periods the cells were harvested, washed, and extracted with trichloracetic acid, and analysed by the Schneider technique as used by Morse and Carter. Irradiated cells held in the basal medium at 18° C show no net synthesis of deoxyribonucleic acid or acid-soluble components and a reduced rate of synthesis of protein and acid-insoluble ribonucleic acid. On the other hand, irradiated cells incubated

at 18° C in yeast extract show essentially normal synthesis of all components (Fig. 9). To date, the results do not permit one to decide whether these alterations in nucleic acid synthesis are the cause or the result of the viability changes in the irradiated population. Further control of growth conditions—for example, the division cycle—might accentuate the recovery phenomenon. Synchronization of division might very well produce an essentially homogeneous population for

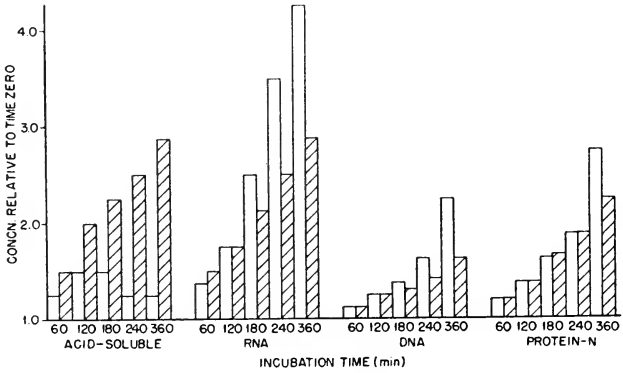


FIG. 9. Relative rates of synthesis of nucleic acids and protein by irradiated (▨) and non-irradiated (□) *Esch. coli* on yeast extract at 18° C.

The acid-soluble fraction represents that fraction of the cells soluble in cold 10 per cent trichloroacetic acid; only ribose was estimated in this fraction by orcinol test.

recovery studies. Such studies are just beginning in the overall programme of bacterial recovery.

Mention should be made of the results of our studies on mammals, i.e., chemical protection during irradiation and posttreatment with bone marrow or spleen. AET will raise the LD_{50} 30 days for $C_3H \times 101$ mice from 692 to 1148 r. Bone marrow will raise the LD_{50} to 1292. A combination of both treatments will bring the LD_{50} to 1863 (Congdon, Upton, and Doherty, 1956, in preparation). Survival after very high exposures (2400 r of gamma-rays) can be obtained if, in addition to the combination treatment, daily injections

of streptomycin are given and possibly some nutritional support is supplied for the irradiated animals (Burnett and Doherty, 1955).

A few generalizations in regard to the mechanism of protection by these different treatments are in order. Chemical protection is apparently quite general, affecting many different functions of the organism. This is obvious from the ability of the chemicals to protect against graying and somewhat against cataract formation, to maintain body weight, and to reduce the number of glandular disturbances. Posttreatment with spleen or bone marrow, apparently, will stimulate the function of the haematopoietic systems. Many interesting findings could be discussed here in regard to the protection by posttreatment, especially the immunological aspects. These may come to light in the discussion. It should be pointed out that most of these studies emphasize only the immediate effects. Very little is known in mammals about the effect of radiation protection on the long-delayed effects; i.e., late-appearing malignancies and mutational changes. Actually, these latter ones may prove to be the most important ones. In bacteria, the situation is somewhat clearer since genetic effects in micro-organisms may be readily observed. This brings up one of the most important questions in modern radiobiology; namely, "Is the genetic damage entirely dependent on the amount of ionizing radiation to which the cell is exposed, or can the effect be modified by chemical protection or by treatment after the exposure has ended?" Bacterial cells are very well suited for checking this since the chemical used will penetrate to most parts of the cell and, after treatment, will help a large number of cells to survive. In a number of strains of *Esch. coli* it has been observed that, for certain mutations (nutritional reversions), the mutation rate increases inversely to the survival ratio and is not necessarily proportional to the amount of radiation to which the cells are exposed. This means that the dose-reduction factor for mutations approaches the DRF for survival. In other words, it is possible in certain strains of

Esch. coli to protect against mutation production by chemicals or recovery factors. However, it is possible to recognize this only if there is a minimum of population pressure effect. Where the population pressure effect is very great, as in reversion of a tyrosineless strain of *Esch. coli*, this population pressure will obscure the entire phenomenon of protection and recovery. In contrast to this, the argininеless strain shows decreased mutation rate with increased survival (Hollaender, Billen, and Doudney, 1955). It is important to point out that the recognition of this phenomenon requires careful analysis for each individual mutation. Very similar results have been obtained in regard to chromosome breaks in bean roots, where it has been possible to modify the number of chromosome breaks by chemical treatment after exposure (Wolff and Luippold, 1955). I hope this will be discussed at a later meeting where cytological effects will be brought up. In any case, it is fairly safe to say that the possibility exists of the modification of genetic effects by radiation protection.

REFERENCES

ALEXANDER, P., BACQ, Z. M., COUSENS, S. F., FOX, M., HERVE, A., and LAZAR, J. (1955). *Radiation Res.*, **2**, 392.

ALEXANDER, P., CHARLESBY, A., and ROSS, J. (1954). *Proc. roy. Soc.*, **223A**, 392.

BACQ, Z. M., HERVE, A., LECOMTE, J., FISCHER, P., and BLAVIER, J. (1951). *Arch. int. Physiol.*, **59**, 442.

BASHFORD, R. E., and HUENNEKENS, F. M. (1955). *J. Amer. chem. Soc.*, **77**, 3878.

BURNETT, W. T., Jr., and DOHERTY, D. G. (1955). *Radiation Res.*, **3**, 217.

BURNETT, W. T., Jr., STAPLETON, G. E., MORSE, M. L., and HOLLAENDER, A. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 636.

CALVIN, M. (1954). Glutathione, A Symposium, p. 3. New York: Academic Press.

CONGDON, C. C., UPTON, A. C., and DOHERTY, D. G. (1956). In preparation.

DOHERTY, D. G., and BURNETT, W. T., JR. (1955). *Proc. Soc. exp. Biol., N.Y.*, **89**, 312.

DOHERTY, D. G., and SHAPIRA, R. (1956). In preparation.

DOUDNEY, C. O., and HOLLAENDER, A. (1956). In preparation.

HOLLAENDER, A., BILLEN, D., and DOUDNEY, C. O. (1955). *Radiation Res.*, **3**, 235.

Hollaender, A., and Doudney, C. O. (1955). Radiobiol. Symposium, p. 112, London: Butterworth's Scientific Publications.
Hollaender, A., Stapleton, G. E., and Martin, F. L., (1951). *Nature, Lond.*, **167**, 103.
Kluyskens, P. (1953). *C.R. Soc. Biol., Paris*, **147**, 733.
Morse, M. L., and Carter, C. E. (1949). *J. Bact.*, **58**, 317.
Stapleton, G. E., Billen, D., and Hollaender, A., (1953). *J. cell. comp. Physiol.*, **41**, 345.
Stapleton, G. E., Sbarra, A. J., and Hollaender, A. (1955). *J. Bact.*, **70**, 7.
Stapleton, G. E., and Woodbury, D. H. (1955). Presentation at regional meeting of Kentucky-Tennessee Branch of the Society of American Bacteriologists, Nashville, Tennessee, November, 1955.
Wolff, S., and Luippold, H. E. (1955). *Science*, **122**, 231.

DISCUSSION

Stocken: Have you any long-term survival in the mixed treatment, say comparing the thiouronium compound plus bone marrow alone or against the thiouronium compound alone?

Hollaender: These experiments are going on now and as far as we have gone (these have been going on for about six months), the mice are all alive. This is if one uses isologous bone marrow; I have not discussed the question of homologous or heterologous bone marrow. This is a different problem and I hope that Dr. Loutit will bring some of these questions up.

Laser: Dr. Hollaender, how do mice react to injection of thiouronium? Do they seem unaffected or are they in a prostrate condition similar to that frequently obtained in mice on application of protective doses of cysteine, which produce pulmonary oedema and a high degree of anaerobiosis?

Secondly, I should like to refer to the notation used in your figures where the ordinates are marked "survival." This is generally taken to mean that the graphs indicate the percentage kill. This, however, is by no means the case. Irradiated cells are not dead but appear to be quite normal in many respects. They have, however, lost the ability to form visible colonies within, say, 48–72 hours, although a certain percentage of them may do so at a later period. In view of the importance of your pioneer work, especially on the oxygen effect, for radiobiology in general and lately also in connection with food technology, and in order to avoid confusion in the literature, may I suggest that you substitute "viable count" for "survival".

One further point: you describe *recovery* from radiation damage, e.g. on addition of an amino acid. Recovery, to my mind, implies the gradual disappearance of an induced and quantitatively determined inhibition and necessitates the measurement of rates. Have you done so or do you mean recovery to be synonymous with less inhibition?

Hollaender: In answer to your first question, I gave you the toxic level for thiouronium. Routinely we use 6 mg. per standard mouse. We get very good protection at 4·4 mg. If you go to 8–10 mg. the mouse will be prostrate and a good percentage will die. The range in which it is safe to use this compound is considerably greater than with cysteamine or cysteine.

Now in regard to your second question, on survival of these bacteria: we emphasize in all our reports that we have determined the survival on the basis of colony-forming organisms. We are very cautious on this point because, as Dr. Stapleton has shown earlier, during discussion after your paper, these bacteria are not dead when they are unable to form visible colonies. They still can respire for a considerable time. As to the question of whether we get more colonies after 72 hours, we check this very carefully. We usually make two counts; for instance when we study the effects of lower temperatures the plates stay for 24 hours at 18° and then for at least 24 hours at 37°. The delayed effect on B/r, i.e. the appearance of late colonies, is very small if X-rays are used. Now if you go to the long u.v. you may have to wait as long as five days before all colonies appear, but that is another point.

Alper: The same thing happens with *Esch. coli* B. I have checked this in some, not all, experiments and found no more colonies after 48 than after 24 hours.

Hollaender: If you use u.v. it is a different story, you have to be more careful there.

Stapleton: With regard to Dr. Laser's question on why we call it recovery, you can define recovery in many different ways and it is apparently prevention of some lesion that would ordinarily lead to death. It could be a lack of an inhibition.

Laser: You could call it prevention rather than recovery.

Stapleton: Yes.

Van Bekkum: Dr. Hollaender, you have commented in your paper on the protective effects of some *S*-alkyl and *N*-alkyl derivatives of cysteamine. Dr. de Groot and I have recently studied a few similar compounds with regard to their protective activity *in vivo* (mice) and *in vitro* (isolated rat thymocytes). The compounds referred to are the following:

(1) *S*-ethylcysteamine:—$C_2H_5 \cdot S \cdot CH_2 \cdot CH_2 \cdot NH_2$
(2) *N*-ethylcysteamine:—$HS \cdot CH_2 \cdot CH_2 \cdot NH \cdot C_2H_5$
(3) *N*-diethylcysteamine:— $HS \cdot CH_2 \cdot CH_2 \cdot N(C_2H_5)_2$

In vivo the *S*-ethyl derivative (1) has some protective activity, which is less than that of cysteamine. The *N*-ethyl derivative (2) is more toxic and less protective than cysteamine, the *N*-diethyl compound (3) is still more toxic to mice and has practically no protective activity *in vivo*. On the other hand, in the thymocyte system both compounds (2) and (3) afford considerable protection, while the *S*-ethyl derivative (1) is completely ineffective. It seems, therefore, that the amine group is most important for *in vivo* protective activity, while the sulphydryl group seems to be required for *in vitro* protection.

We have also investigated a number of amines, which are known to protect mice excellently, like histamine, epinephrin and phenylethylamine, and it was found that these compounds do not show any protective activity with isolated cells. We think that the protection of mice by these amines may be the result of their activity on some organ system e.g. the cardiovascular or the respiratory system, and it seems possible that part of the protective activity of cysteamine *in vivo* is due to a similar mechanism. Have you any information on the pharmacological effects of cysteamine?

Hollaender: Yes, we have. This is work which has been done at the University of Rochester, it is not completed and that is why I didn't mention it. AET as well as cysteamine have a depressive effect on the respiratory centres in the cat. AET is more toxic to the dog than to the mouse. Preliminary tests in monkeys have shown that AET is less toxic to the monkey than to the dog. The effect is different in different species of animals. This, of course, requires very careful investigation before we would say it is of practical significance.

Spiegelman: I should like to cite here an interesting experiment performed by Norman (1953, *J. Bact.*, **65**, 151) which is relevant to some of the points raised by Dr. Hollaender's paper. Norman grew cells in a glucose minimal medium, irradiated them with u.v., and then plated them on minimal media containing glucose in some cases and other carbon sources in others. On plotting the log of the survivors measured on these different plates, he found a much greater apparent kill if the cells were plated on a carbon source different from the one on which they had grown up. Thus, glucose-grown cells show a much greater kill when plated on lactose as compared with glucose. That this apparent increase in sensitivity is associated with a demand for the formation of new enzyme is shown by experiments in which he grew the cells up in lactose and plated them on lactose minimal medium. In such cases the apparent kill was the same as when plated on glucose.

Latarjet: This is u.v. work—it destroys the adaptation.

Spiegelman: Yes, but the interesting thing to emerge from this experiment is the following. First, it must be noted that all you are asking the glucose cell to do, on being plated on lactose, is to make one more enzyme, and this apparently makes an enormous difference. In addition, when the killing curves obtained from the glucose and the lactose plates are extrapolated back, they both give the same average hit number required for kill and the value is about 1·2. This would suggest that a nuclear phenomenon is involved. It would seem to me that a more extensive investigation of this phenomenon is extremely desirable. It is an experimental situation in which the comparative conditions on the glucose and lactose minimal media are under experimental control and where the enzymatic consequences are also pretty well defined.

Haddow: Dr. Hollaender, have you done any tests on protection against carcinogenesis with AET?

Hollaender: These are in progress now. Many of these malignancies appear fairly late, it takes a period of about two years to be sure that

they don't have any. Dr. Upton in our laboratory is now maintaining a large colony of mice which have survived this irradiation. We are watching for leukaemias and also for other malignancies. But it may take another year to be certain.

Laser: I have tried it with yeast and have obtained quite conclusive results but with opposite effect. Yeast is grown in a synthetic medium containing all the growth requirements, with glucose and ammonium sulphate as the only sources of carbon and nitrogen. This yeast, when grown in the same medium after irradiation, shows a strong irradiation effect. However, if the irradiated yeast is transferred into a medium in which the ammonium salt is replaced by another nitrogenous substrate to which the cells may have to adapt, the radiation effect is diminished and still more so if the glucose is also replaced by a different carbon source, say galactose.

Spiegelman: I don't understand. What do you plate on?

Laser: I don't plate, but grow the yeast in a liquid medium and measure growth rates either by optical or manometric methods. I find, as already stated, that by changing after irradiation either the carbon or the nitrogenous source or both—that is, by initiating adaptation—the irradiation effect has become less apparent.

Spiegelman: Your killing is less?

Laser: Yes.

Popjak: I would like to raise some questions about the mechanism of action of these chemical protectors. It has been said, in the case of cysteamine for example, that it might be that it protects the "business-end" of coenzyme A. Then it has been suggested that it acts like cysteine and other readily oxidizable substances by virtue of taking up preferentially oxidizing radicals, or by reducing tissue oxygen tension, this being merely some kind of nitrogen effect. It seems to me that there might be some other explanations for the protective action of cysteamine. It appears that the free amine group is very important in protection, because substitution on the nitrogen with alkyl groups eliminates the protective action. Eldjarn and Pihl have reported that cysteamine does combine very rapidly with the SH groups of proteins. If that is so, then one might expect that cysteamine and analogous substances might cause a reversible inhibition of enzymes. I have done some experiments on this point and these support my assumption. It may be that cysteamine protects enzymes during the period of irradiation by combining with some vital groups on the enzyme, rather than by catching oxidizing radicals.

Hollaender: It just keeps the radicals from getting to a group by protecting them?

Popjak: Yes.

Gale: There seem to be a number of analogies between the material that we have just been given and the action of penicillin. I noticed particularly one of the slides that Dr. Hollaender showed, where there appeared to be an effect of radiation resulting in decreased synthesis of RNA, and increased accumulation of the "acid-soluble RNA". I wonder if you can tell us anything about the nature of substances which

in fact form the so-called "acid-soluble RNA" in these irradiated organisms.

Stapleton: No, we have not looked at this, but we certainly intend to do so, especially on the basis of some of the work that has been done with u.v., which indicates that irradiated cells pile up DNA precursors. The point Dr. Gale refers to is the only indication in all these experiments that there is any pile-up of anything.

Gray: In support of what Popjak has just been saying, Burns did an experiment with yeast in which he obtained quite appreciable protection with *p*-chloromercuribenzoate which did not, however, eliminate the oxygen effect. The oxygen effect was still shown in the protected cells. I think in the case of cysteine we have got to be extremely careful in any given case to be sure that it is not operating simply by removal of oxygen from the solution.

Whether or not cysteine protects mammals by inducing a state of tissue anoxia I do not know, but an examination of cases reported in the literature in which the addition of cysteine to solutions in which bacteria were suspended or roots immersed reveals that the experimental conditions were in each case such as to produce partial or complete anoxia at the time of irradiation.

Popjak: The first oxidation product of cysteine would be cystine. There are some enzymes which can reduce disulphides very effectively and very rapidly, the typical example, of course, being the glutathione reductase which has been described in wheat germ and pea seedlings, but which Dr. Hele and I find to be present also in animal tissues and which has a high specificity for TPN. So even though it may get oxidized, in the cells it will get reduced again to cysteine.

Gray: I am not speaking of possible chemical mechanisms of cysteine protection, that is quite beyond me; I am just saying that before the occasion for a chemical theory arises we must first be sure that we are not dealing with simple anoxia in any given case. This has very often not been established.

Hollaender: May I say that I don't want to leave the impression that we have been able to reverse mutations. All we believe that we have been able to do is to stop the effect before the mutation has been carried through, before the damage has been completed. But what we have done with these chemicals, either before or especially after irradiation, is to interfere with the completion of the mutation process.

POSTIRRADIATION TREATMENT OF MICE AND RATS

D. W. H. Barnes and J. F. Loutit
Medical Research Council Radiobiological Research Unit, Harwell

From time to time there appear reports that specific treatments given to mice after an acute lethal dose of radiation have a significant effect in improving the proportion of survivors at the conventional time of 30 days. Amongst the chemical agents alleged to have this therapeutic activity have been batyl alcohol (DL-α-octadecylglycerol-ether) (Edlund, 1954); properdin (a natural euglobulin present in normal serum) (Pillemer *et al.*, 1954) polyvinylpyrrolidone (Bürger *et al.*, 1954) and carbon monoxide (Konecci *et al.*, 1955). Up to date none of these reports has been followed by confirmatory papers. In the case of polyvinylpyrrolidone the beneficial effect has been denied (Becker and Kirchberg, 1955; Bürger, 1955; Rugh *et al.*, 1953).

On the other hand, procedures which might be called surgical are undoubtedly effective. For instance Brecher and Cronkite (1951) showed that lethally irradiated rats could recover if, after their irradiation, they were subjected to the operation of parabiosis with an unirradiated rat. Following this operation a cross-circulation between the two animals is established. The survival of the damaged animal may be due to the transfer of anything in the blood of the normal animal. It could be due to the continuous transfusion of the formed elements of blood—red corpuscles, leucocytes and platelets—all of which are grossly deficient in the circulation of the rat some days after a lethal dose of radiation. However, blood transfusion as can be practised clinically has been without effect except for the limited successes claimed on small series by Salisbury and co-workers (1951) and Allen and co-workers (1951). For such trials the rat is not suitable as the experi-

mental animal and the dog is the animal of election. Moreover the results of Brecher and Cronkite can equally well be attributed to the transfer of some factor in solution or colloidal suspension in the plasma exchanged. Such a substance could "detoxify" the damaged animal—an hypothesis which did not appeal to the authors—or act as a stimulant for that early regeneration of the recipient's bone marrow which was observed. This histological finding by itself suggests that the effect was more than that expected from the symptomatic treatment of continuous transfusion with whole blood. Still another suggested alternative was the possibility of a transfer of blood-forming cells from the donor.

Another surgical procedure has been much more widely investigated. Jacobson and his colleagues (1949) first showed that irradiation of mice with their spleens protected was much less lethal than complete irradiation of the whole body plus spleen. They proceeded to demonstrate (1951) that, in similar fashion, a reduced mortality of the totally irradiated mouse could be attained by implanting intraperitoneally spleens from normal mice, accelerated regeneration of the haemopoietic tissues being identified as the fundamental effect of this procedure. Similar, dramatic changes in mortality were reported by Lorenz and his colleagues (1951, 1952) following the injection of suspensions of bone marrow into irradiated mice. Jacobson (1952), in a comprehensive review of the work of his group and of Lorenz and colleagues, marshalled the evidence in favour of his hypothesis that a humoral factor in normal spleen and bone marrow (both haemopoietic tissues in the mouse) was responsible for stimulating the recovery of the irradiated animal's damaged haemopoiesis. Only one argument seemed to the present authors to be incontestably in favour of this hypothesis, namely that heterologous material from guinea pigs had, in the hands of Lorenz and colleagues (1952), been effective. The principle was confirmed later by Congdon and Lorenz (1954) who obtained positive results using bone marrow from rats.

Further evidence in favour of the humoral hypothesis

came later from San Francisco. Cole and co-workers (1952) showed that homogenization, with its attendant severe damage to cells, did not destroy the therapeutic effect. Furthermore, fractionation of these homogenates in sucrose by ultracentrifugation (Cole *et al.*, 1953) localized the effective principle in the layer of greatest density composed of cell nuclei (and a few whole cells). The activity of this nuclear fraction could be destroyed by preparations made from crystalline desoxyribonuclease and trypsin (Cole and Ellis, 1954) which were said to be without action on intact cells (Cole and Ellis, 1955).

Against the humoral theory we have argued on the following grounds.

(1) On repeating the original observations of Jacobson and co-workers, we were impressed that, in those mice which survived the critical month but died subsequently, some of the spleens implanted had "taken" and had become accessory spleens.

(2) We confirmed that an intact organ was not an essential, that suspensions were effective on injection, and that the intravenous route gave results superior to the intraperitoneal (Barnes and Loutit, 1953). (We were not at that time able to obtain improved survival from heterologous material from guinea pigs or rabbits.) This superiority of the intravenous route would not be expected if a soluble hormone were involved but would be in the case of cells.

(3) When we adopted intravenous injections as a routine we noted that there was an approximate threshold of material above which no further improvement was obtained. Jacobson and co-workers (1955) have made similar observations. Depending on the material used this threshold may be 10^5–10^6 cells.

(4) The active principle is extremely thermolabile: it is inactivated in a few minutes at 50°C (Cole, Fishler and Ellis, 1955) and in a few hours at room temperature, at 4° C and at − 15° C (Barnes and Loutit, 1954). This is

compatible with its being cellular. To test this further we have adopted one of the schedules recommended for the preservation of living cells (Smith, 1954), i.e. storage of the material in glycerol at — 79° C, and found that the activity is preserved for 80 odd days at least (Barnes and Loutit, 1955).

(5) The activity is destroyed by a dose of a few hundred röntgens of X-rays *in vitro* (Cole *et al.*, 1953) and *in vivo* (Barnes and Loutit, 1954) which again is more in favour of its being of a cellular rather than a chemical nature.

(6) In most laboratories it is not possible to keep the survivors of animal experiments for the rest of their lives. We have had sufficient accommodation to allow us to do so. We have thus accumulated information on the overall survival of the normal unirradiated CBA mice of our colony (these are not strictly controls in the temporal sense), and mice irradiated with 950 r and treated with isologous (CBA) spleen, spleen from homologous strain A mice and heterologous bone marrow from Wistar rats. The median survival time of the unirradiated mice is 900 days. For the irradiated animals only those which survive the conventional 30 days are included for scoring. Routinely they come to experiment at about the age of 100 days. The median survival time for those given isologous spleen is a further 400 days and for those given homologous spleen approximately 40 days. From the limited data for heterologous transfer, the survival seems much the same as for homologous.

(7) The previous result suggests that antigenic differences, such as occur between cells of various origin, are important. We have another similar observation in that CBA animals, previously immunized by intravenous injections of tissues from mice of strain A, will no longer recover following irradiation with 950 r if treated with spleen from strain A (Barnes and Loutit, 1954).

However, until an accredited cell-free material is shown to be potent, or until cells from the donated material are shown unequivocally to repopulate the host, there can be no absolute confirmation of either hypothesis.

Two sets of recent observations from the Radiobiological Research Unit allow us now to be dogmatic that, unlikely as it originally seemed, repopulation from the donor does in fact occur.

1. Mitchison (1956) used the inbred strains of mice, A and CBA, with which our previous work had been done. CBA male mice of about three months of age were irradiated as in previous experiments with 950 r X-rays (240 kv.; 15 ma.; HVL = 1·2 mm. Cu; dose rate 43 r/min.). This dose is almost invariably lethal to untreated mice. On the same day, after irradiation, the CBA mice were injected intravenously with a suspension of cells from the spleen of infant mice of strain A. The fresh spleens were cut into small pieces, suspended in fresh rabbit serum and macerated with an electrically driven mincer. Each mouse received 0·4 ml., the equivalent of two-fifths of a spleen—that is about 15×10^6 cells estimated from counts made in a haemocytometer. These mice were sacrificed at intervals and tissues were taken for test of their content of antigens specific for strain A.

The method of test involved administration of the respective tissues to normal CBA mice. Each tissue for test was macerated and injected intraperitoneally. If it contained, in adequate numbers, cells derived from strain A it would thereby induce in the normal CBA mouse, within a period of 8–12 days, the state of transplantation immunity. The injected CBA mouse was, after the interval of 8–12 days, inoculated subcutaneously with a suspension of tumour cells specific for mice of strain A—sarcoma 1 (Dunham and Stewart, 1953). This tumour had been maintained in the ascites form by repeated passage in strain A mice. Ascites fluid was diluted with isotonic sodium citrate until it contained approximately 10^6 cells in the 0·1 ml. used for injection. The reaction was scored after a further 8 days when the tumour, or its remnant, was carefully excised and weighed.

In control tests carried out in parallel with the experiments it was shown that CBA mice, previously inoculated with tumour to give full transplantation immunity, on re-injection with the test-dose of tumour returned weights for the implants which varied from individual to individual and from batch to batch within the range of 2 to 42 mg. On the other hand normal, non-immunized CBA mice, while also varying between individuals and between batches, had tumours weighing 48–877 mg. Within batches there was in each case a clear-cut difference, usually one to two orders of magnitude, between individuals of the positive control group and individuals of the negative control group. The weights of the tumours derived from the test-animals were compared with these controls. The tumour-weight in an experimental animal was taken to be significantly different from weights of the non-immune controls if it was less than the mean minus twice the standard deviation.

In addition to the tissues, spleen, lymph nodes and liver, the tissue-fluids, blood and peritoneal exudate, were also assayed for the antigen of strain A. Peritoneal exudates were induced by prior injection of sterile paraffin and consisted of lymphocytes and mononuclear cells.

The results of the tests for A antigen in the tissues and tissue-fluids of the irradiated CBA mice treated with intravenous injections of strain-A spleen are shown in Table I, overleaf. These figures show that the spleen and lymph glands of these animals usually give significantly positive results from the earliest time tested—4 days. The positive results persist until the latest time of test—51 days. Peritoneal exudates also gave positive results throughout the time of test, 16–51 days. On the other hand liver and blood, tested only at 14 days, gave negative results.

Similar tests were conducted with tissues from unirradiated CBA mice injected intravenously with suspensions of spleen from strain-A mice. The results are given in Table II, overleaf. These results indicate that the injected A cells may

Table I

Results of Tests for Strain-A Antigen in Tissues and Tissue-fluids From CBA Mice Irradiated with 950 r X-rays and Promptly Injected Intravenously with a Suspension of Strain-A Spleen.

Fractions indicate the number of positive results out of the total.

Interval after irradiation and injection	*Tissue or tissue-fluid tested*				
	Spleen	*Lymph nodes*	*Peritoneal exudate*	*Blood*	*Liver*
4 days	2/2	4/5	—	—	—
7 days	2/5	3/5	—	—	—
13 days	—	4/5	—	—	—
14 days	4/5	3/5	—	0/5	0/5
16 days	—	—	2/2	—	—
19 days	4/5	—	1/3	—	—
36 days	—	—	2/2	—	—
51 days	5/6	5/6	2/2	—	—

persist and perhaps multiply in the unirradiated CBA mouse for a few days only.

In ancillary experiments it was demonstrated that the critical dose of materials from mice of strain A to give positive results was: peritoneal exudate, 10^5 cells; suspension of spleen, 10^6 cells. It is possible that the trauma to cells attendant on

Table II

Results of Tests for Strain-A Antigen in Tissues and Tissue-fluids from Unirradiated CBA Mice Injected Intravenously with a Suspension of Strain-A Spleen

Fractions indicate the number of positive results out of the total.

Interval after injection	*Tissue or tissue-fluid tested*		
	Spleen	*Lymph nodes*	*Blood*
4 days	3/5	0/5	0/5
7 days	1/5	—	—
14 days	0/5	—	0/5
53 days	0/5	—	—

making the suspension of spleen cells accounts for the increased number neccssary compared with the exudate which could be obtained without damage to the cells.

Whereas 10^6 strain-A spleen cells are necessary to produce a state of transplantation immunity, in the experiments above 15×10^6 spleen cells were injected intravenously into the CBA mice. It is possible that these cells were favourably distributed in the normal or irradiated recipient and accounted for the positive results recorded for tissues in the first days after injection. However, in the unirradiated animals these positive results were soon reversed to negative as was expected on the basis of immunity following the implant of foreign and incompatible tissue. In the irradiated animals the positive results not only persisted in tissues that were manifestly and measurably enlarged and hyperplastic, but even showed evidence of increase for which the original paper should be consulted. Moreover, it is noteworthy that a peritoneal exudate induced at a late stage was also positive.

One must conclude that the persistence of the A antigen against all the laws of tissue grafting is a result of the massive dose of radiation; and that the apparent increase in A antigen is the result of the growth of the grafted strain-A cells or the incorporation of the A antigen by the CBA host. Recent work from other laboratories leads to the same conclusions. Main and Prehn (1955) demonstrated that mice injected with homologous cells after lethal doses of X-rays survived and would then take skin grafts which normally would be incompatible. Lindsley, Odell and Tausche (1955), using rats, could identify red blood cells characteristic of the donor in rats irradiated with near lethal doses and treated with homologous bone marrow. Finally, Nowell and co-workers (1955) have reported that mice injected with rats' bone marrow develop myeloid cells and circulating leucocytes which give positive histochemical reactions for alkaline phosphatase—a property of the rat but not of the mouse.

2. The rather improbable explanation involving incorporation of the donor's antigens into the host need no longer be

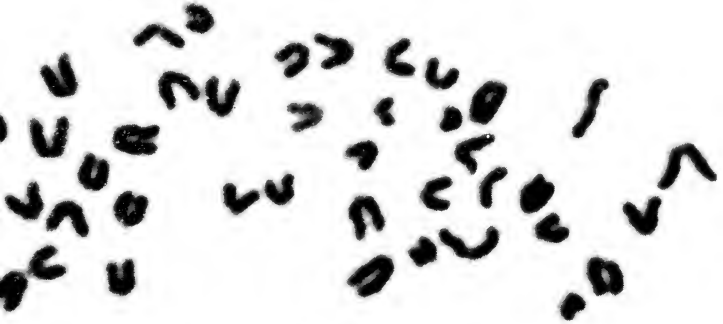
invoked in view of the second line of approach to be reported. This is the use of marked chromosomes in the donated material. Two different markers have been employed (Ford *et al.*, 1956).

(I) *The rat chromosomes.* It has already been noted that administration of heterologous material can allow the lethally irradiated mouse to survive (Lorenz *et al.*, 1952; Congdon and Lorenz, 1954). We have lately been able to repeat this result using as donors inbred rats from our colony which stems from the Wistar strain. Our CBA mice are given 950 r as before and then injected intravenously with a suspension of bone marrow obtained from the two femurs of a young rat. The recipient CBA mouse has cells with a complement of 40 chromosomes with terminal centromeres (Fig. 1). The rat has cells which contain 42 chromosomes, a number of which have a characteristic cruciform appearance in metaphase (Fig. 2), since their centromeres occupy a central position. In a squash preparation it is therefore easy to differentiate those cells in metaphase which are derived from the host, i.e. the mouse, from those which stem from the donor-rat.

(II) *Mouse-translocation T6.* Carter, Lyon and Phillips (1955), by irradiating the testes of male mice and promptly breeding from them, were able to select offspring that were semi-sterile as a result of inheriting a pair of translocated chromosomes from the irradiated parent. Ford and Hamerton in this laboratory examined cytologically the available stocks carrying these translocations and showed that one (denoted by Carter and co-workers as T6) had a chromosome which was readily differentiated at metaphase from any of the normal chromosomes of the mouse. It was about half the length of the smallest normal chromosome (Fig. 3). Dr. Lyon has bred for us young mice carrying this translocation in the heterozygous state and we have used the spleens of these mice aged 7–10 days as donor material for the irradiated CBA mice.

The irradiated CBA mice, treated with either the rat bone marrow or the spleen of the T6 mouse, have been sacrificed at intervals after irradiation and treatment and their

ash preparations of cells in metaphase of mitosis after colchicine treatment :



. 1. From normal CBA mouse—40 chromosomes, all centromeres effectively terminal.

. 2. From normal Wistar rat—42 chromosomes, some centromeres occupy central position.

[facing page 148

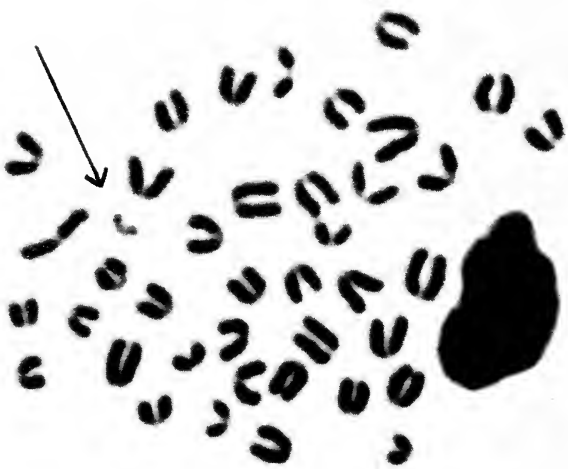


FIG. 3. From T6 mouse with one characteristic ultra-short chromosome due to a translocation.

FIG. 4. From CBA mouse irradiated with 950 r and treated with intravenous injection of spleen from T6 mouse; showing typical damage from radiation, dicentric chromosomes and acentric fragments.

tissues have been examined cytologically by Ford and Hamerton. The technique employed was an adaptation of the Feulgen squash method. The animals were injected with colchicine one hour before sacrifice and the tissues were handled as suspensions.

The results to date may be summarized as follows. Of the bone marrow cells in metaphase which could be scored, the great majority have been identified by means of the marker as originating from the donor (Table III). This holds also for

Table III

PRELIMINARY DATA OF FORD AND HAMERTON ON IDENTIFICATION OF MARKERS IN CELLS AT METAPHASE (+ PRESENT, − ABSENT, ? DOUBTFUL)

Time of sacrifice	*No. of animals scored*	*Cells in metaphase—Identification of marker T6*											
		Bone Marrow			*Spleen*			*Lymph node*			*Thymus*		
		+	−	?	+	−	?	+	−	?	+	−	?
5 days	3	201	1*	104	113	1*	11	21	0	13	28	1*	7
14 days	1	185	1*	13	35	2*	4	—	—	—	45	0	6
28 days	2	114	0	12	140	0	41	62	2*	14	—	—	—
49 days	2	197	0	84	—	—	—	10	0	0	51	0	8
70 days	2	299	0	52	105	0	8	—	—	—	—	—	—
		Cells in metaphase—Identification of marker—rat											
5 days	2	127	4*	0	184	0	0	—	—	—	8	1*	0
11 days	2	549	0	1	—	—	—	—	—	—	—	—	—
19 days	2	695	0	11	—	—	—	—	—	—	—	—	—
28 days	2	219	0	23	929	13	0	205	12	38	436	0	48
49 days	2	739	0	20	—	—	—	—	—	—	—	—	—
49 days	1	0	599	5	4	492	0	16	51	0	—	—	—
55 days	2	163	43	9	—	—	—	—	—	—	—	—	—
65 days	1	0	9	0	7	49	0	—	—	—	—	—	—

* Mostly cells showing classical signs of damage from radiation.

the much more limited examination of spleen, lymph gland and thymus, the only tissues as yet of which satisfactory preparations have been made. For each time of examination

where more than one animal has been sacrificed, the cell-counts reported in Table III are the sum of the individual counts. On the one occasion when one differed from its mate or mates the count on that animal is recorded separately. Preparations were made of liver but no mitoses were seen. Testes were examined but were completely atrophic as judged by the naked eye and without mitotic figures in the cytological preparations.

The exceptions to the broad generalization made above are as follows:

(i) In the process of making the squash some cells are violently disrupted and the full complement of 40 chromosomes of the mouse's cell or 42 of the rat's cell may not be traced. While the rat's cell may be identified by the characteristic cruciform members of the set, even in the absence of a full count of chromosomes, to diagnose with certainty in the homologous transfer the presence or absence of the marker, necessitates the visualization of all 40 chromosomes. In some cells with counts of less than 40 chromosomes the marker was not seen. These cells have been scored as "doubtful".

(ii) Cells may not be sufficiently well spread for all 40 chromosomes to be separated and seen. If the marker is not identified, it is not possible to say whether such a cell is from donor or host and the cell is also recorded as "doubtful".

(iii) Occasional cells are seen in the early days after irradiation with multiple lesions of the chromosomes of the kind well known to be induced by radiation e.g. dicentric chromosomes and acentric fragments. These cells can be attributed to the host, but they do not persist after the first few days (Fig. 4).

(iv) While in the early days and weeks after such irradiation it appears as if the great majority at least of the cells in division are attributable to the donor, it may well be that in the course of time the host's tissues under investigation will show some recovery. Thus, after seven weeks in the case of the heterologous transfer of rat bone marrow to mouse, we

have seen the reappearance of mitoses of murine cells. It is, however, premature to do more than record the findings so far.

In preliminary experiments where less than the 99 per cent lethal dose of 950 r has been given to CBA mice, we see this reversal of cellular types at a comparatively early time after irradiation. When CBA mice are given 545–575 r and treated with T6 spleen, the regenerating tissues are mainly of the T6 type in the first week, but later belong mainly to the normal CBA type. In further preliminary experiments CBA mice were irradiated over only part of the body. The hind third was given 1200 r and T6 spleen was then injected intravenously. The regenerating bone marrow of the femora and inguinal lymph glands at 5 days corresponded in cell-type to the normal CBA host.

The interpretation of these experiments is clear. In the mouse given the LD_{99} of X-rays the regenerating cells, seen in mitosis, of the haemopoietic tissues are almost without exception characteristic of the material from the donor. The living cells in the preparation injected must, therefore, be dividing and colonizing the empty spaces of bone marrow and lymphatic tissue. The alternative explanation of the former experiment of Mitchison—that the host had incorporated antigens—can no longer be maintained. In the homologous transfer it is inconceivable that the host's cells had accepted whole chromosomes (translocations at that), rejected some of its own to maintain a normal complement and still had a balanced set for division. Equally it is unnecessary in the heterologous transfer to postulate complete exchange of chromosomes by the host.

The preliminary results of experiments involving sub-lethal doses of irradiation suggest that the length of the symbiosis of donor's and host's cells may be dependent on the dose of radiation given. It seems that the immune mechanisms which normally determine compatibility of tissue grafts are in abeyance following massive doses in the lethal and supralethal range. This refractoriness is long lasting. From the direct evidence of Table III, we show it has lasted for

ten weeks in the case of acquired tolerance to homologous tissue. From indirect evidence of experiments as yet incomplete we infer that it may be more or less permanent. Chimaeras, formed by irradiation of CBA mice with 950 r and treatment with spleen of strain A, have been kept, as noted, routinely until death. Such animals as were available have been challenged with subcutaneous inoculations of the tumour sarcoma-1; ten, having survived 18 to 246 days after irradiation, have all "taken" the tumour and succumbed.

REFERENCES

Allen, J. C., Moulder, P. V., and Emerson, D. M. (1951). *J. Amer. med. Ass.*, **145**, 704.

Barnes, D. W. H., and Loutit, J. F. (1953). *Proc. R. Soc. Med.*, **46**, 251.

Barnes, D. W. H., and Loutit, J. F. (1954). *Nucleonics*, **12**, No. 5, 68.

Barnes, D. W. H., and Loutit, J. F. (1955). *J. nat. Cancer Inst.*, **15**, 901.

Becker, J., and Kirschberg, H. (1955). *Strahlentherapie*, **98**, 343.

Brecher, G., and Cronkite, E. P. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 292.

Bürger, H. (1955). *Strahlentherapie*, **98**, 348.

Bürger, H., Grabinger, H., and Lehmann, J. (1954). *Strahlentherapie*, **95**, 399.

Carter, T. C., Lyon, M. E., and Phillips, R. T. C. (1955). *J. Genet.*, **53**, 154.

Cole, L. J., and Ellis, M. E. (1954). *Radiation Res.*, **1**, 347.

Cole, L. J., and Ellis, M. E. (1955). *In* Radiobiology Symposium, p. 141. Ed. Bacq and Alexander. London: Butterworth.

Cole, L. J., Fishler, M. C., and Bond, V. P. (1953). *Proc. nat. Acad. Sci., Wash.*, 39, 759.

Cole, L. J., Fishler, M. C., and Ellis, M. E. (1955). *Radiology*, **64**, 201.

Cole, L. J., Fishler, M. C., Ellis, M. E., and Bond, V. P. (1952). *Proc. Soc. exp. Biol., N.Y.*, **80**, 112.

Congdon, C. C., and Lorenz, E. (1954). *Amer. J. Physiol.*, **176**, 297,

Dunham, L. J., and Stewart, H. L. (1953). *J. nat. Cancer Inst.*, **13**, 1299.

Edlund, T. (1954). *Nature, Lond.*, **174**, 1102.

Ford, C. E., Hamerton, J. L., Barnes, D. W. H., and Loutit, J. F. (1956). *Nature, Lond.*, **177**, 452.

Jacobson, L. O. (1952). *Cancer Res.*, **12**, 315.

Jacobson, L. O., Marks, E. K., and Gaston, E. O. (1955). *In* Radiobiology Symposium, p. 122. Ed. Bacq and Alexander. London: Butterworth.

Jacobson, L. O., Marks, E. K., Robson, M. J., Gaston, E. O., and Zirkle, R. E. (1949). *J. Lab. clin. Med.*, **34**, 1538.

JACOBSON, L. O., SIMMONS, E. L., MARKS, E. K., and ELDREDGE, J. H. (1951). *Science*, **113**, 510.
KONECCI, E. B., TAYLOR, W. F., and WILKS, S. S. (1955). *Radiation Res.*, **3**, 157.
LINDSLEY, D. L., ODELL, T. T., and TAUSCHE, F. G. (1955). *Proc. Soc. exp. Biol., N.Y.*, **90**, 512.
LORENZ, E., CONGDON, C. C., and UPHOFF, D. (1952). *Radiology*, **58**, 863.
LORENZ, E., UPHOFF, D., REED, T. R., and SHELTON, E. (1951). *J. nat. Cancer Inst.*, **12**, 197.
MAIN, J. M., and PREHN, R. T. (1955). *J. nat. Cancer Inst.*, **15**, 1023.
MITCHISON, N. A. (1956). *Brit. J. exp. Path.*, **38**, 239.
NOWELL, P. C., COLE, L. J., HABERMEYER, J. G., and ROAN, P. L. (1955). USNRDL-TR-59.
PILLEMER, L., BLUN, L., LEPOW, I. H., ROSS, O. A., TODD, E. W., and WARDLAW, A. C. (1954). *Science*, **120**, 279.
RUGH, R., SUESS, J., and SCUDDER, J. (1953). *Nucleonics*, **11**, No. 8, 52.
SALISBURY, P. P., REKERS, P. E., MILLER, J. H., and MARTI, N. F. (1951). *Science*, **113**, 6.
SMITH, A. U. (1954). *Proc. R. Soc. Med.*, **47**, 57.

DISCUSSION

Latarjet: I would like to add a comment to Dr. Loutit's paper. It seems to me that at the present state of our knowledge there is a critical question which concerns this 50 days: does the proliferation of homologous or even heterologous material still occur at 50 days because the immunity is still down, or does it occur because a state of tolerance has been established by adding this homologous material when the immunity has been broken down? If we were to wait longer, could we reach a state where full immunity has been recovered and the homologous cells still occur, or not? Do total body irradiations transform an adult into a newborn animal?

Loutit: I think it is probably a bit too early to be dogmatic; but at any rate we have had some of these homologous animals from our previous work as long as eight months after receiving homologous tissue, and at eight months after receiving homologous tissue they will still take tumours specific to the donor and not the host. Main and Prehn's skin graft work was of about the same duration. I think the foreign skin graft survived up to 190 days.

Hollaender: Our mice have received rat bone marrow; we have quite a number now which have been kept for 160 days, the erythrocytes are still full red. What usually happens is that about 50 per cent die between 30 and 60 days, another 25 per cent apparently get some kind of a disease. The 25 per cent which then survive seem to be as permanent as we can have (this work has been done by Dr. Makinodan in our laboratory).

Haddow: Do you know the cause of death?

Hollaender: The first 50 per cent probably died because they finally

produced antibodies against the rat bone marrow. We have no explanation for the next 25 per cent. They lose weight, some go down to 9 g. and then die.

Van Bekkum: I'm very glad that Dr. Loutit has been able to demonstrate so admirably that his original point of view with regard to the mechanism of bone marrow therapy is the correct one. I should like to mention some of our results in this field which are in essential agreement with Dr. Loutit's findings. The point is that we have employed quite different techniques to identify the origin of the haematopoietic cells in mice which survived a lethal dose of total body irradiation as a result of homologous or heterologous bone marrow injections. These experiments were carried out in collaboration with Dr. O. Vos, Mr. J. A. G. Davids and Mr. W. W. H. Weyzen. The methods are summarized in Table I. The first technique is based on the observation that

Table I (Van Bekkum)

EVIDENCE FOR THE CELLULAR HYPOTHESIS

Combination	*Surviving mice, identification of*	*Method*
Homologous	Bone marrow	Therapeutic effect in irradiated donor and receptor strain
Heterologous	Erythrocytes	Specific antisera
Heterologous	Granulocytes	Histochemical

isologous bone marrow cells are about 20 times more effective than homologous bone marrow cells when injected into lethally irradiated CBA mice (Fig. 1). In other words, you need about 20 times more

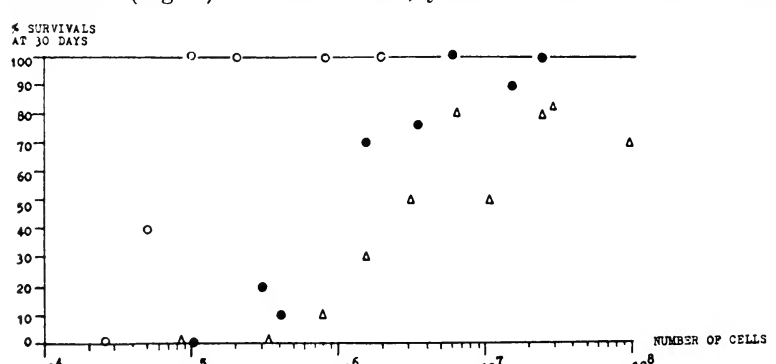


FIG. 1. (Van Bekkum). Therapeutic effect of i.v. bone marrow cells in irradiated mice.

○ Isologous. ● Homologous. △ Heterologous (rat).

(nucleated viable) homologous cells than isologous cells to obtain the same therapeutic result. We wanted to know whether the regenerated bone marrow of irradiated C57 Bl. mice, that had been treated with CBA bone marrow cells, consisted of CBA cells or of C57 Bl. cells. The question could be answered by injecting this bone marrow into both

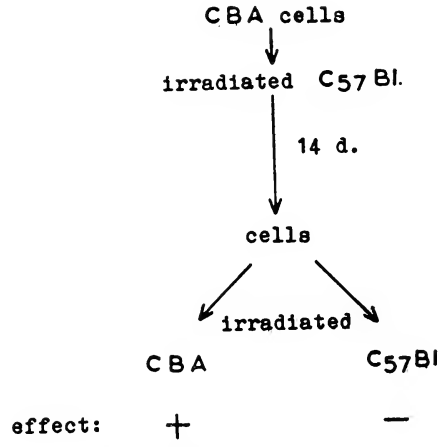


FIG. 2. (Van Bekkum).

irradiated CBA and C57 Bl. mice and by comparing the therapeutic effects in the two strains (Fig. 2). The results of this experiment (Table II) strongly suggest that the bone marrow tested consisted predominantly of CBA cells.

Table II (Van Bekkum)

THERAPEUTIC EFFECT OF REGENERATED BONE MARROW

Number of cells $\times 10^5$	*% survivals at 32 days*	
	CBA	*C57Bl*
1	0	0
2	30	0
4	100	0
8	90	0

The second method employed specific antisera, which permitted the identification of rat and mouse erythrocytes. In this case lethally irradiated CBA mice were injected with rat (WAG) bone marrow cells and agglutination reactions were carried out with both antisera in samples of the peripheral blood of the survivals. Fig. 3 shows that from the tenth day after irradiation onward a gradual replacement of the

mouse erythrocytes by rat erythrocytes occurs. These results also demonstrate that the injected rat bone marrow has survived and proliferates in the irradiated mice. Results of a similar nature have been reported by Lindsley, Odell and Tausche (1955, *Proc. Soc. exp. Biol., N.Y.*, **90**, 512) in the case of homologous bone marrow injection into irradiated rats, although in these experiments no significant effect of the bone marrow on survival was observed.

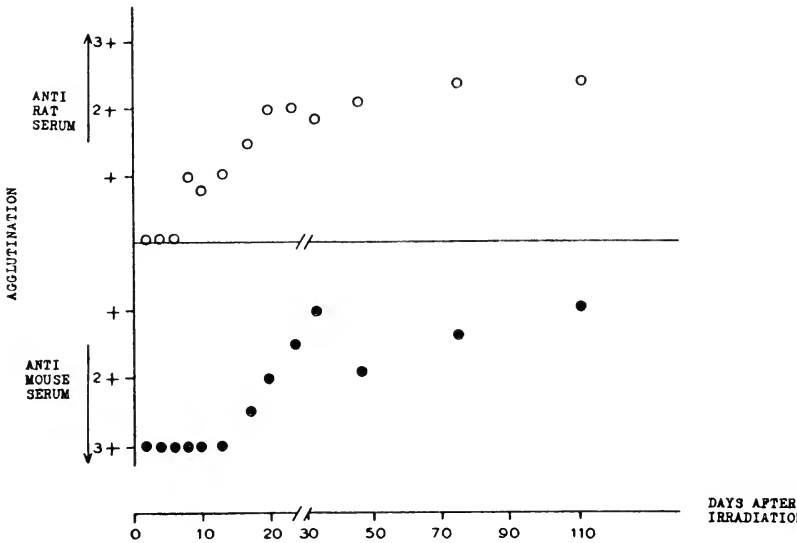


FIG. 3. (Van Bekkum). Identification of erythrocytes in irradiated and rat bone marrow treated mice.

In our third approach a histochemical method was used which permits differentiation between rat and mouse granulocytes. Wachstein (1946, *J. Lab. clin. Med.*, **31**, 1) observed that rat granulocytes show a strongly positive alkaline phosphatase reaction while mouse granulocytes are consistently negative in this respect. Irradiated mice were injected with rat bone marrow and on various days after the irradiation smears of the peripheral blood were prepared and studied with the alkaline phosphatase reaction. Table III shows the percentage of positive granulocytes in irradiated mice after treatment with rat bone marrow. In most cases the granulocytes were predominantly identified as rat cells. It should be noted that Nowell, Cole and Habermeyer (1955, USNRDL report T. R. 59) have independently obtained identical results using Wachstein's method.

Finally, I want to comment on the survival of irradiated mice after treatment with homologous and heterologous bone marrow. As shown

Table III (Van Bekkum)

ALKALINE PHOSPHATASE REACTION IN GRANULOCYTES

	% positive cells
Control mice	0
Control rats	99
Irradiated and rat bone marrow treated mice	
46th day after irradiation	> 94 (13) 17 (1)
76th day	> 93 (8) 0 (1)
111th day	> 97 (4) 68 (1) 0 (1)
170th day	71 (1) 86 (1)

The figures in brackets represent the number of observations.

in Table IV, there is practically no delayed mortality in the case of isologous bone-marrow therapy, but after homologous and heterologous therapy a large percentage of the mice die between the thirtieth and the sixtieth day after irradiation. These data are also in agreement with those of Dr. Loutit, although I think that our 100-days survival rate is slightly better.

Table IV (Van Bekkum)

DELAYED MORTALITY OF IRRADIATED AND BONE MARROW TREATED MICE

Bone marrow	*Number of survivals*			
	30 *d.*	60 *d.*	80 *d.*	100 *d.*
Isologous	34	33	33	33
Homologous	39	25	19	18
Heterologous (rat)	42	15	15	13

Spiegelman: I might note first that I consider the transformation hypothesis quite unlikely. However, it seems to me that your test for its existence is insensitive, because you are demanding that the transformation result in a complete conversion of the chromosomal apparatus, and this is highly improbable. We know that in bacterial transformations only a minute portion of the chromosomal material is actually incorporated, and the number of characters involved is invariably small, one or two. It would be better then to look at somewhat more restricted changes in phenotype, for example, perhaps with respect to the antigenic properties of the cell. A simple experiment perhaps is to

take rat cells which have established themselves in a mouse and introduce them into a normal, non-irradiated mouse and see if they disappear. Has this been done?

Van Bekkum: Not yet.

Spiegelman: I think this would test it very cleanly and directly.

Lajtha: Can these irradiation and bone marrow transfusion experiments be repeated before the mice die, in those mice which die, say after 50 days? Those mice which die only after 30 days will show some of their original cells returning, which probably means that their immune reaction starts killing off the donor cells. Now, could a repeat irradiation and transfusion then be performed and thus elongate the life of the animal? Furthermore, do you think that such a treatment could "cure" an experimental leukaemia in the animals?

Loutit: I suppose it could, but I have not done it, and I don't know what the answer would be.

Stocken: I would like to show a slide which is relevant to Cole's proposition that nuclear material is concerned in the curative factor. This slide (Table I) shows decreasing numbers of whole cells and varying

Table I (Stocken)

30 DAYS SURVIVAL OF CBA MICE AFTER 950 r X-RAYS AND I.V. INJECTION OF SPLEEN FRACTIONS

Nuclei $\times 10^6$	*Whole cells* $\times 10^3$	*Survival*
—	60	4/4
6·5	60	4/4
—	50	3/4
1·3	50	3/4
—	36	3/4
6·0	36	4/4
24	24	1/4
2·2	10	0/20
1·0	1·4	0/20
36	$< 0{\cdot}6$	0/10
10	$< 0{\cdot}2$	0/10

amounts of nuclei. I think it is reasonably clear that when the number of whole cells is reduced to less than 30,000, the recovery does not take place. You will notice that where 36,000,000 nuclei were put into the mice no benefit was obtained.

Hollaender: I would like to keep an open mind with regard to cell-free extracts still possibly producing recovery. Even if you establish the bone marrow and have tissue growing in the animal, the recovery process as such may be a product of something which is given up by the cells. I think we should keep searching, and I think some of the work which Dr. Stapleton has done may be pertinent at this point.

Stapleton: While we were studying prevention of death or recovery in bacteria, we had some indication that precursors for RNA synthesis might be involved. We wondered if an RNA polynucleotide fraction from *Esch. coli* would stimulate recovery in bacteria. We prepared a

rather crude polynucleotide RNA fraction from *Esch. coli* and found it was stimulatory in recovery of *Esch. coli*. We tried this same preparation injected into irradiated mice, and it seemed to work in mice too. These experiments are extremely preliminary, but it looks as if the same sort of preparation injected repeatedly into animals might bring about something of the order of 20 per cent survival in mice irradiated with a supralethal dose. We have had a lot of variability among samples prepared similarly. Some fractions have been toxic. We hope that when we iron out some of the difficulties concerning toxicity of some of this material prepared from bacteria, we can then give a better answer than we can at the present time. There is no possibility here, of course, of repopulation.

Stocken: I think that Dr. Loutit used very careful but accurate words when he said that the humoral factor was not the initiating mechanism. I think that this completely covers it.

Stapleton: One reason why I hesitated to say anything about this bacterial fraction was the fact that people have found increased survival somewhat like we have found by injecting materials such as ground glass, charcoal, and so on, following irradiation.

Butler: Then what is the status of Cole's nucleoprotein fraction? Is the effect due to cells?

Loutit: With Stocken we have made preparations according to the Cole recipe, using one of these marker chromosomes and the recovering marrow in those animals contains the marker. That suggests to me either that the residual number of whole cells are the effective thing, or if it is the nuclei, that they can reform their own cytoplasm and get cracking again from that. I find that a little far-fetched.

Stocken: In self-defence, I think that this preparation here has been reasonably well done. All the cells have been counted. We have used various techniques for looking at nuclei, and I don't think that Cole had these advantages. I do think that his material was probably contaminated with whole cells.

Alexander: An impressive argument which Cole used was that he said that his "nuclear" preparations could be inactivated by treatment with DNAse, whereas the cellular preparations could not. This would show that the activity of the "nuclear" preparations is not due to contamination with whole cells, since these should not be inactivated by DNAse. But Dr. Jacobson told me last year in Chicago that although DNAse does not inactivate preparations containing large numbers of cells (of the order of several million), it does destroy the activity of suspension containing 10^4 or so cells per ml. Since this number of cells is sufficient to produce appreciable effects and is also the amount of contamination to be expected in preparations of nuclei, the DNAse test can no longer be considered as decisive proof for the nuclear hypothesis.

Stocken: We have done these experiments too, on the same preparation and there is no recovery with the DNAse-treated preparation.

Spiegelman: Have these cells been stained to see whether they still have DNA material?

Stocken: No. They have been examined by phase contrast microscopy

in 28 per cent protein solution as described by Baner, Joseph and Esnouf (1955, *Science*, **123**, 24).

Stapleton: Someone in Jacobson's group did a very nice experiment using leukaemia cells, making a nuclear fraction, and from the nuclear fraction they were able to transplant leukaemia to another animal. I think they found also that the leukaemia cells were acted upon by the same enzymes that Cole proposed were not effective on whole cells. This seems to me to be good proof that there were cells in the nuclear fraction, plus the fact that the enzymes did work on some whole cells.

Stocken: Yes, Jacobson came to the conclusion that he needed 100,000 cells.

STUDIES ON THE MECHANISM OF PROTEIN SYNTHESIS*

P. C. Zamecnik, Elizabeth B. Keller, M. B. Hoagland, J. W. Littlefield and R. B. Loftfield

Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University, Massachusetts General Hospital, Boston

The investigation of protein synthesis received a great stimulus ten years ago, when the ready availability of radioactive isotopes made it possible to pursue in greater detail the line of research initiated by the Schoenheimer school (Schoenheimer, 1942). After a few fruitful years, however, this approach reached a plateau. During the past two or three years there has again occurred great progress in this field, due to the application of other techniques—centrifugal, microbiological, and cytochemical—to the problem. Two good reviews (Tarver, 1954; Borsook, 1956) have appeared recently, covering early as well as current work in the field.

Brachet (1941) and Caspersson (1941) first called attention to the high concentration of ribonucleic acid in the cytoplasm of cells of rapidly growing tissues, and in cells of tissues engaged in the secretion of proteins. The work of Palade (1955) and of Sjöstrand and Hanzon (1954) has utilized the high resolving power of the electron microscope to call attention to details of structure of the fine reticular network found in the cytoplasm of many cells. Two principal constituents of this cytoplasmic network are (1) the double-walled membranes, and (2) the small dense granules visualized in high concentration both on the exterior walls of the tubules, and also as unattached particles. These structures have been particularly evident in secretory cells such as those of the pancreas.

* Supported by grants in aid from the U.S. Atomic Energy Commission and the American Cancer Society. This is a publication of the Harvard Cancer Commission.

Work in this laboratory has become confluent with this cytochemical stream as a consequence of our interest in particulate cell constituents concerned in protein synthesis. In agreement with the initial observation of Borsook and coworkers (1950), we have found the microsome fraction of the rat liver cell to be that centrifugally separable component of the cytoplasm most rapidly labelled with ^{14}C-tagged amino acids (Keller, Zamecnik and Loftfield, 1954).

By means of sodium deoxycholate it has been possible to separate the two above-mentioned principal components of the "microsome" fraction, and to determine that the small, dense granule (ribonucleoprotein particle) has a higher specific radioactivity in its protein than does the vesicular or fragmented membranous component (Littlefield *et al.*, 1955). The careful work of Palade and Siekewitz (1956) in tracing the lineage of these microsomal constituents back to the small, dense granules and membranes of the cell serves as a bridge between cellular topography and cell fractionation studies. Our conclusion about the high rate of labelling of the ribonucleoprotein particle is based on experiments which were carried out on whole rats. Their distinctive features were (1) the intravenous injection of ^{14}C-leucine or ^{14}C-valine and (2) the employment of labelling times of 2–10 minutes. These conditions brought out maximal differences between the specific activities of proteins located in the various cell fractions, differences which became less distinguishable with increased time periods.

These studies also suggested that the ribonucleoprotein particle *in vivo* was engaged in a rapid turnover process, in which protein or large peptide fragments synthesized therein were passed on to other parts of the cell. It has been calculated (Littlefield and Keller, unpublished) that the rate of labelling of the cytoplasmic ribonucleoprotein particles of the liver is sufficient to account for most of the protein synthesis of the rat liver. This is not to imply that protein synthesis may not occur also in the nucleus, but it does strengthen the thought that the main pathway of synthesis of a protein

molecule may pass through the cytoplasmic ribonucleoprotein particle.

In parallel with these *in vivo* studies, we have investigated the incorporation of labelled amino acids into proteins in cell-free fractions of rat liver. One has little hesitancy in calling the *in vivo* work a study on the mechanism of protein synthesis. In the cell-free preparations, however, there is no net protein synthesis as measured by the usual procedures, and the term "incorporation" encompasses this degree of uncertainty. In our context, however, this term "incorporation" does have a rather precise meaning, which begins with the choice of labelled amino acid. On the basis of past experiments in our own laboratory (Zamecnik and Frantz, 1949) and of others (cf. Tarver, 1954), we consider it safest to employ labelled amino acids which are least likely to engage in other known metabolic processes of the cell. For example, glutamic acid, aspartic acid and alanine are located at the gateway to the citric acid cycle; glycine is a precursor of the purine molecule, and is readily converted to serine and phosphatidyl serine; lysine may become bonded to protein in side-chain linkage by its ε-amino group (Schweet, 1955); and the sulphur-containing amino acids can attach to protein by disulphide bonding (Tarver, 1954). The possibility that glutathione is being synthesized in a cell-free system is further reason for avoiding when possible glutamic acid, cysteine, and glycine as amino acid labels for experiments on incorporation into proteins. No doubt in time a particular pitfall will be found for the use of every amino acid as a labelled precursor for protein.

These considerations, however, have led us to the general use of L-leucine and L-valine in our recent incorporation experiments (Loftfield and Harris, 1956). Partial hydrolysis of labelled protein at the end of typical *in vitro* experiments has indicated the presence of a variety of labelled peptides (Zamecnik and Keller, 1954; Keller and Zamecnik, 1956; Zamecnik *et al.*, 1956), and provides evidence of alpha-peptide bonding.

We have several types of evidence bearing on the question

of whether this peptide bonding represents *de novo* synthesis of a peptide chain or exchange of a single amino acid for its non-radioactive isotope within the interior of an existing peptide chain. To begin with, Littlefield and Keller (1956) have shown that biologically active microsomes, labelled by incubation of ascites tumour cells with ^{14}C-leucine, do not lose their protein label when incubated in a complete cell-free system containing 0·01 M ^{14}C-L-leucine. In another type of experiment, liver microsomes, labelled by 3-minute cell-free incubation with ^{14}C-leucine or ^{14}C-valine of high specific activity, did not lose this radioactivity when ten times as much inert leucine or valine was added (Littlefield *et al.*, 1955) for a further incubation period. These specific pieces of data argue against a simple exchange reaction as the mechanism of labelling. Reasoning along more general lines, the hydrogen bonding and specific three-dimensional patternization of a completed protein molecule would appear to prohibit exchange of a single amino acid for another located in the interior of the peptide chain. The data are more compatible, therefore, with the conception that the labelled amino acids measure a small amount of *de novo* synthesis of long chain peptides in the ribonucleoprotein particulate fraction of this cell-free system. During the 10-minute cell-free incubation at 37° in which the labile ribonucleoprotein particle retains its biological activity, up to 0·2 per cent labelling of the ribonucleoprotein leucine occurs.

There are five essential components of the cell-free incorporation system: (1) the microsome fraction, (2) the pH 5 precipitable enzyme fraction, (3) ATP (and usually an ATP-regenerating system), (4) GTP or GDP, and (5) the labelled amino acid. The general method of preparation of the protein fractions is indicated in Fig. 1. If any one of these constituents is omitted, the incorporation suffers (Keller and Zamecnik, 1956). It has recently been possible (Littlefield and Keller, 1956) to simplify this system a little by using cellular fractions prepared by 0·5 M-NaCl extraction and centrifugal fractionation of distilled water lysates of Ehrlich

mouse ascites tumour cells. Here it is possible to obtain good incorporation into the ribonucleoprotein particulate fraction of the microsomes, in the almost complete absence of the membranous lipoprotein fraction. While these two fractions of the microsome pellet can also be separated by means of sodium

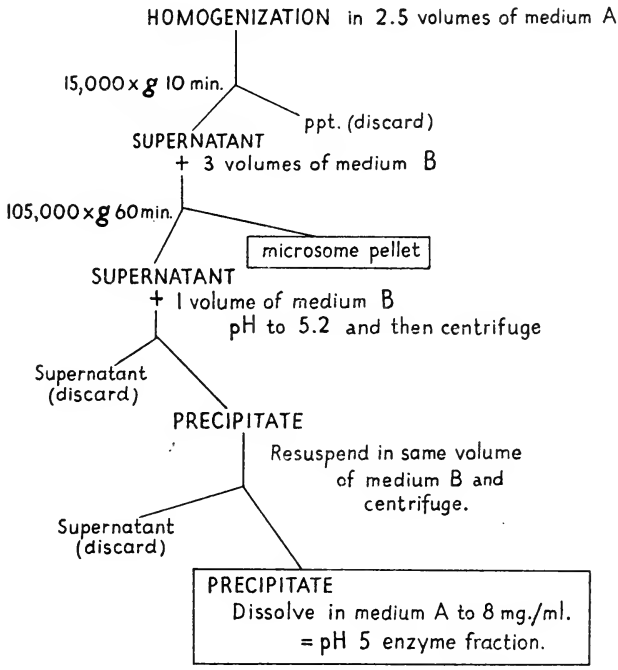


FIG. 1. Fractionation scheme for rat liver.
Medium A: 0·35 M sucrose, 0·035 M-$KHCO_3$, 0·004 M-$MgCl_2$, 0·025 M-KCl.
Medium B: 0·9 M sucrose, 0·004 M-$MgCl_2$, 0·025 M-KCl.

deoxycholate, after such treatment the ribonucleoprotein particles are biologically inactive, presumably due to denaturation. This newer separation method is a gentler procedure, which preserves the fragile synthetic activity of this particle.

Another advantage in using cellular fractions from the mouse ascites tumour is that 10 μM of ATP per ml. in itself is adequate for energy generation, and the ATP regenerating

system (phosphopyruvate plus pyruvate kinase) may be omitted; however, GTP (or GDP) is still required.

An amino acid activating mechanism has recently been found which is catalysed by the "pH 5 enzyme" fraction of the rat liver protein in the presence of ATP (Hoagland, 1955; Hoagland, Keller and Zamecnik, 1956). The reaction appears to proceed as follows:

$$\text{ATP} + \text{amino acid} + \text{enzyme} \rightleftharpoons (\text{aminoacyl}\cdot\text{AMP})\ \text{enzyme} + \text{pyrophosphate}$$

The evidence points to several separate activating enzymes, rather than to a single enzyme or activation site capable of activating all the amino acids.

The rôle of GTP (or GDP) in this incorporation process is puzzling. It has thus far been unable to substitute for ATP in Hoagland's amino acid activation reaction. GTP has been found to be an essential cofactor for the incorporation of all six labelled amino acids tested (Keller and Zamecnik, 1956). It is a very specific cofactor, the only active one among many nucleoside tri-, di- and monophosphates tested. Through the kindness of Dr. Waldo Cohn we were able to test the following dinucleotides of guanine, obtained from partial hydrolysis of RNA and possessing a free 3′-phosphate: GC, CG, GU, UG, GA and AG. All of these compounds, in roughly 0·1 μM per ml. concentration, were unable to substitute for GTP. Addition of 0·25 mg. of AGUC ribonucleotide polymer, kindly furnished by Dr. Severo Ochoa, to the *in vitro* test system caused a slight inhibition of the incorporation. In order to test for the GTP requirement, it is necessary to use a pH 5 enzyme fraction washed quite free of endogenous GTP, and to employ a microsome fraction prepared by centrifugation of a diluted 15,000 × ***g*** supernatant fraction of rat liver homogenate (Keller and Zamecnik, 1956).

Our present conception of the sequence of events in the process of protein synthesis as observed in rat liver cytoplasm is summarized in Fig. 2. The rôle of the ribonucleoprotein particle is considered to be the sequentialization of activated

amino acids. In the schemes suggested by Dounce (1952) and by Koningsberger and Overbeek (1953), the amino acids are attacked by covalent bonding of the amino or carboxyl group respectively of the amino acid with the phosphate of the ribonucleic acid. Since, however, the amino acids appear to be activated in the soluble enzyme fraction of the cell, it would be reasonable to consider that activated aminoacyl nucleotide compounds line up along a ribonucleoprotein

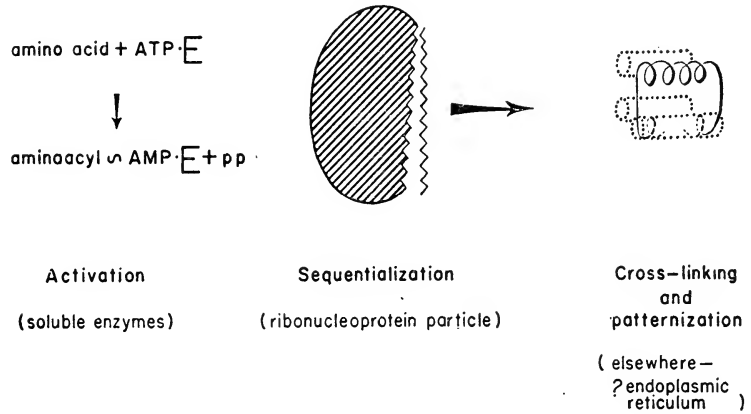


FIG. 2. Postulated steps in protein synthesis in rat liver cytoplasm.

template, with their side-chain R groups determining the sequence by their ability to fit into particular spaces occurring on the ribonucleoprotein surface, rather than that there occurs a formal triester linkage of amino acid to nucleic acid. Van der Waal's forces and electrostatic charges would serve as the binding forces of the side chains of the activated amino acids to the ribonucleoprotein template. This scheme has been drawn up in detail by our colleague Loftfield, and will be published elsewhere.

In the *in vivo* experiments of Littlefield and co-workers (1955), the ribonucleoprotein fraction of the liver cell appears to pass on its radioactive protein or large peptide chain to

other fractions of the cell. This is particularly so for the membranous, lipid-rich, deoxycholate-soluble portion of the microsome fraction. In electron micrographs of intact liver cells, these membranes are in close juxtaposition to the ribonucleoprotein particles, where they may serve as acceptor for a formed peptide chain, and as a site for its transformation into a completed protein molecule or lipoprotein complex.

Our evidence suggests therefore that protein synthesis in the rat liver cytoplasm may be divided into three steps, as indicated in Fig. 2. Further subdivisions remain tasks for the future.

REFERENCES

BORSOOK, H. (1956). *J. cell. comp. Physiol.*, **47**, Suppl. 1, p.35.

BORSOOK, H., DEASY, C. L., HAAGEN-SMIT, A. J., KEIGHLEY, G., and LOWY, P. H. (1950). *J. biol. Chem.*, **187**, 839.

BRACHET, J. (1941). *Arch. biol. (Liège)*, **53**, 207.

CASPERSSON, T. O. (1941). *Naturwissenschaften*, **28**, 33.

DOUNCE, A. (1952). *Enzymologia*, **15**, 251.

HOAGLAND, M. B. (1955). *Biochim. biophys. acta*, **16**, 288.

HOAGLAND, M. B., KELLER, E. B., and ZAMECNIK, P. C. (1956). *J. biol. Chem.*, **218**, 345.

KELLER, E. B., and ZAMECNIK, P. C. (1956). *J. biol. Chem.*, **221**, 45.

KELLER, E. B., ZAMECNIK, P. C., and LOFTFIELD, R. B. (1954). *J. Histochem. Cytochem.*, **2**, 378.

KONINGSBERGER, V. V., and OVERBEEK, J. TH. G. (1953). *Koninkl. Nederl. Academie Van Wetenschappen Proc.*, Series B, **56**, 248.

LITTLEFIELD, J. W., and KELLER, E. B. (1956). *Fed. Proc.*, **15**, 302.

LITTLEFIELD, J. W., KELLER, E. B., GROSS, J., and ZAMECNIK, P. C. (1955). *J. biol. Chem.*, **217**, 111.

LOFTFIELD, R. B., and HARRIS, A. (1956). *J. biol. Chem.*, **219**, 151.

PALADE, G. E. (1955). *J. biophys. biochem. Cytol.*, **1**, 59.

PALADE, G. E., and SIEKEVITZ, P. (1956). *J. biophys. biochem. Cytol.*, **2**, 171.

SCHOENHEIMER, R. (1942). The Dynamic State of Body Constituents. Harvard University Press.

SCHWEET, R. (1955). *Fed. Proc.*, **14**, 277.

SJÖSTRAND, F. S., and HANZON, V. (1954). *Exp. Cell Res.*, **7**, 393.

TARVER, H. (1954). *In* The Proteins, Vol. II, Part B, p. 1199. New York: Academic Press.

ZAMECNIK, P. C., and FRANTZ, I. D., JR. (1949). *Cold Spr. Harb. Symp. quant. Biol.*, **14**, 199.

ZAMECNIK, P. C., and KELLER, E. B. (1954). *J. biol. Chem.*, **209**, 337.

ZAMECNIK, P. C., KELLER, E. B., LITTLEFIELD, J. W., HOAGLAND, M. B., and LOFTFIELD, R. B. (1956). *J. cell. comp. Physiol.*, **47**, Suppl. 1, p. 81.

DISCUSSION

Work: We too have been following uptake of radioactive amino acids into liver preparations in the intact animal, and have adopted a similar centrifugal fractionation scheme followed by a scheme of salt fractionation of the guinea pig liver microsomes. We found also that we had peak activity in the microsomes. A nucleoprotein fraction obtained by salt fractionation of the microsome material showed a quite clearly defined peak thirty minutes after injection; we could separate out two nucleoprotein fractions, one of which showed this peak of activity and one which did not. Why there should be a peak at thirty minutes in our guinea pigs and a peak at a very much shorter period in Dr. Zamecnik's rats, I don't know. It looks as though we are both handling very similar types of material.

On the additional steps in the reaction, the activation of the carboxyl group, we have been able to confirm Hoagland's work. Hoagland very kindly wrote and gave us details before he published this.

I was interested in your remarks about the use of leucine AMP anhydride; we have made alanine AMP anhydride, and have found that it is unstable; in aqueous solution it polymerized rapidly to its peptide. I would be interested in hearing if anybody had managed to get a sufficiently stable preparation to work with. It seems to me that the amino group must be protected in some way or other in the enzymic carboxyl activation process. As you say, it may be attached to the enzyme surface in some way which protects the amino group. Another distinct possibility, I think, which is worth consideration, is that the amino group may also be protected by a phosphoramide linkage. We made some phosphoramides and found that metabolically they were indistinguishable from the free amino acids; in other words the phosphoramide group obviously comes off very quickly in a biological system, and it seems to me that it is a possible intermediate which we ought to consider.

Another point that I think we ought to keep in mind is that these mixed anhydrides are so unstable that if you add any acceptor which is potentially capable of forming an anhydride group, even in the absence of any enzyme, you get mixed anhydride. Thus if you take AMP and, say, leucine phosphate anhydride you get ATP from it without any enzyme.

Zamecnik: De Moss, Genuth and Novelli have just published (1956, *Fed. Proc.*, **15**, 241) their results, and their yield of this leucyl AMP anhydride was very poor, about 7 per cent. The half-life of this compound was several minutes at pH 7, making possible a study of its conversion to ATP by the enzyme in the presence of pyrophosphate. With regard to your work on guinea pigs, the technical details of our experiments with the rat and yours with the guinea pig may be a little different. I suppose we both injected intravenously?

Work: Yes.

Zamecnik: In one type of experiment we gave a small dose of ^{14}C-leucine of a very high specific activity, about 9 mc per m-mole (1·4

mc to a 270-g. rat), in the hope that we would get a high level of radioactivity incorporated into protein in a few minutes (cf. Fig. 3, Littlefield *et al.*, 1955, *loc. cit.*).

Work: We may have missed a peak which you picked up in using a higher dose. We used about 35 mc/kg. animal. Ours was a comparable specific activity, but a much smaller dose.

Zamecnik: As regards the two different ribonucleoprotein fractions, in the case of the rat liver we obtained particles such as this using sodium deoxycholate fractionation. Ultracentrifugally, the ribonucleoprotein fraction behaves as one peak, although I don't think that necessarily implies very much. But when my colleagues Littlefield and Keller prepared ribonucleoprotein particles from the Ehrlich mouse ascites tumour they found three closely parallel ribonucleoprotein peaks. I don't know whether the specific activity in those were all the same.

Work: We also found that if we fractionated the supernatant with ammonium sulphate we got very considerable variation in activity.

Zamecnik: We have carried out some fractionation of this 100,000 *g* supernatant protein too. Dr. Hoagland has rather good evidence that there is not just one enzyme or one enzymatic site involved in activation of all amino acids. I don't know whether there is one enzyme for each amino acid, but there is evidence for three or four separate ones so far. De Moss and Novelli (1955, *Biochim. biophys. acta*, **18**, 592) have confirmed this finding in bacteria, and they found evidence for activation of 8 separate amino acids, and think there is more than one enzyme involved.

Work: That would fit in with our experience. Whenever we fractionate we divide it into a lot of fractions with less activity than the original.

Zamecnik: We have not had good luck in fractionating this pH 5 enzyme and then using these fractions separately for the whole incorporation process. We seem to lose the activity on fractionation, and my impression is that we have several enzymes involved, which are going into different fractions.

Popjak: Is it not possible that the aminoacyl nucleotide or aminoacyl AMP is only a first intermediary so to speak, and that the hydroxamate that you get in these preparations perhaps really comes from another type of activated amino acid; in the acetate activation reaction it is also postulated that it is the acetyl AMP which is the first intermediate and then acetyl coenzyme A is formed. I think that this is very likely, particularly in view of what Dr. Work has said about the instability of mixed anhydrides of amino acids with AMP.

Zamecnik: That fits in with the idea that maybe one has an enzyme-bound activated intermediate.

Work: One point I should mention: we found that the rate of appearance of ATP is very much faster than the rate of appearance of hydroxamate. Does that agree with your findings?

Zamecnik: Yes, our preparations contain our "ATPase", unrelated to amino acid activation, in addition to the ATP splitting involved in this process.

Popjak: It is very likely that you have more than one activating

enzyme, because even in the case of simple homologues like fatty acids several activating enzymes are known, all of which have different chain-length specificity; so that in a group of more diverse substances like amino acids it is even more likely that you should have more than one activating enzyme.

Brachet: Do you know (a) whether the protein present in these very small particles has any of the enzymatic activity which is usually associated with microsomes; (b) whether it is something like a histone or any of the basic proteins; and (c) whether removal of the ribonucleic acid from these very small granules has an inhibitory effect on incorporation?

Zamecnik: We have added ribonuclease in very small concentrations, about 1 μg./ml., and have found it inhibits the incorporation reaction completely. There is some breakdown of RNA, but the inhibition appears to exceed it enormously. We have done some preliminary fractionation experiments on these particles. If we add ribonuclease at a concentration of 5 or 10 μg./ml. and incubate it with these particles for 10–15 minutes, everything comes down in a coagulum, all stuck together. We were disappointed with that experiment. But we have also incubated ribonucleoprotein particles with between two-tenths and 1 μg. of ribonuclease in a cold room at 4°C for 3 days, in the presence of a fairly high salt concentration; under those circumstances the ribonucleoprotein broke down into several fragments. A fraction which did not centrifuge down at 100,000 *g* for 2 hours had about twice the specific radioactivity of the fraction which did spin down. This soluble protein fraction was placed in the electrophoresis machine (we have only done this once). There were three components. We cut between the slowest moving peak and the other two peaks and found that the slowest moving peak had half the specific activity of the others. That is a crude fractionation, but it suggests that we may be able to break this ribonucleoprotein down by some procedure. I have no doubt that it consists of more than one protein. We suspect that only a small portion of the protein components is involved in the rapid synthetic mechanism.

Holmes: I should like to know if Dr. Zamecnik has any evidence of the RNA itself being broken down and rebuilt during amino acid incorporation.

Some years ago we prepared a crude cytoplasmic ribonucleoprotein fraction from the tumours of rats injected during life with ^{35}S-methionine and ^{32}P. This fraction was prepared by precipitation at pH 5·0 after removal of the nuclei and contained all the remaining RNA and a considerable amount of protein. The uptake of ^{32}P into the RNA seemed to parallel the incorporation of methionine into the protein. X-ray irradiation of the tumour *in vivo* had no effect upon the uptake of either of the labels, whereas the injection of shock-producing chemicals caused a proportionate reduction in the uptake of both.

Zamecnik: In Potter's laboratory experiments were performed on *in vitro* incorporation of labelled orotic acid into RNA in rat liver homogenates; they used almost the same components that we have used here, and found that the orotic acid does make its way into the RNA molecule.

We have not done definitive experiments in our laboratory, but I suspect, from the few we have carried out, that RNA is being synthesized, since ^{14}C-labelled ATP makes its way into RNA during that same time.

Spiegelman: Along these lines it seems to me very worth while, in order to define the nature of this system, to enquire more closely into the relation between nucleic acid synthesis and protein. As you know, in the study of enzyme formation there seems to be no doubt that there is a mandatory coupling between RNA synthesis and synthesis of the new protein molecule. It would be interesting to see whether for example an agent like hydroxyuridine would affect this incorporation phenomenon as it does the synthesis of the protein molecule. Dr. Zamecnik, in your *in vivo* incorporation experiments have you ever simultaneously injected a complete mixture of amino acids, in addition to the labelled one, to see whether there was any effect?

Zamecnik: We have injected a "quenching" dose of the inert amino acid and then one has a drop off.

Spiegelman: The point of that experiment is to see whether, if you make protein synthesis much easier, your incorporation will also increase.

Zamecnik: There is an adequate intercellular supply of free amino acids in the whole animal, isn't there?

Spiegelman: There is a supply, but is it adequate?

Zamecnik: But you can calculate that it is adequate for about 5–10 minutes of protein synthesis in the rat liver.

Spiegelman: The yeast cell has a very high internal supply too but you can stimulate protein synthesis in yeast cells immediately and considerably by providing an external supply of amino acids. I should also like to know whether you have tried a reconstitution experiment of your ribonuclease-treated material.

Zamecnik: Yes, we have tried this type of experiment; it doesn't work.

Pirie: Have you got any evidence of intermediate peptides of any size, large or small?

Zamecnik: No. My colleague Dr. Loftfield has been specially interested in that question and has been studying ferritin synthesis in the rat liver. If you give colloidal iron oxide to a weanling rat, the weanling rat synthesizes 10 mg. of ferritin within the next two days, whereas previously he had none. That is a case of *de novo* synthesis of protein. The specific activity of leucine or valine in the ferritin is approximately the same as that of the intracellular free leucine or valine concentrations, and Loftfield concludes that the ferritin molecule is not supplied with any appreciable amount of peptide fragments from the large amount of liver protein already existing, but that the ferritin molecule appears to be formed from free amino acids exclusively. Furthermore, in the amino acid activating system there is no evidence that amino acids can react with an activated amino acid to form a peptide.

I might mention that it is still possible to preserve a modicum of doubt as to whether there is a real distinction between "exchange" and "synthesis" in amino acid incorporation experiments. It is hard for us to make a distinction in the animal system we use. We seem to be

dealing with what we interpret to be *de novo* synthesis. I think one could even say that in dealing with bacterial systems, where one does not add a complete complement of amino acids, there may be enough residual amino acids to provide a complete supply of amino acids for a short time, whereas over a longer period of time they would eventually be exhausted. There is a certain amount of proteolysis going on also. One may define "exchange" as a simultaneous opening of two peptide bonds in the interior of a long peptide chain with a substitution of another amino acid. I wonder whether that does in fact happen.

Work: I feel fairly convinced on purely chemical grounds that it does not. Once the peptide bond is formed it is a completely different order of stability and it is extremely unlikely that it would open again.

NUCLEIC ACIDS AND AMINO ACID INCORPORATION

E. F. Gale

Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge

In order to investigate the biological functions of nucleic acids, it is necessary to devise preparations of cells in which it is possible to modify the nucleic acid components without affecting other factors involved in relevant biochemical activities. Although the actions of transforming principles are demonstrated in growing cultures, intact cells in general do not appear to be permeable to nucleic acids and, consequently, the actions of the latter must be studied in subcellular preparations. Since the demonstration that suitable preparations could be obtained from broken staphylococcal cells (Gale and Folkes, 1953*a*), a number of other subcellular materials have been obtained in which direct investigation of nucleic acid function is possible (Allfrey, 1954; Beljanski, 1954; Lester, 1953; Littlefield *et al.*, 1955; Nisman, Hirsch and Marmur, 1955; Nisman, Hirsch, Marmur and Cousin, 1955; Webster and Johnson, 1955; Zamecnik and Keller, 1954). As a result of investigations with these preparations it has been found that the incorporation of labelled amino acids is dependent upon the presence of nucleic acids and that, in appropriate structures, the process can be mediated by ribo- and by deoxyribonucleic acid (Gale, 1956*a*). The purpose of this contribution is to discuss what we ourselves have learned of the function of nucleic acids in amino acid incorporation studied in disrupted staphylococcal cells, and consists largely of a summary of material recently published elsewhere (Gale and Folkes, 1955*a* and *b*; Gale, 1956*a* and *b*).

Disrupted Staphylococcal Cell Preparation

If a suspension of *Staphylococcus aureus* is incubated in the presence of glucose and a single amino acid, such as glutamic acid, labelled with ^{14}C, the protein of the preparation acquires radioactivity. This radioactivity is not removed by prolonged washing of the protein fraction with trichloracetic acid, acetic acid, alkali, alcohol, ether or acetone and can be released from the protein fraction only by such chemical measures as result in hydrolysis of the peptide bonds. If the amino acid in the incubation medium is glutamic acid, hydrolysis and separation of the protein residues after incubation show that the radioactivity is confined to the glutamic acid residues. Treatment of the protein with ninhydrin or by application of Sanger's method for N-terminal groups (Sanger, 1945) shows that less than 3 per cent of the incorporated residues occur as end-groups. It seems that the situation is essentially similar to that studied by Borsook and his colleagues (reviewed by Borsook, 1954) in other tissues, and that the incorporated radioactive amino acid has become part of the structure of certain proteins of the preparation.

If cells rendered radioactive, by incubation with glucose and a labelled amino acid, are disintegrated by exposure to supersonic vibration and the disintegrated material then separated on the centrifuge into four fractions: (a) material sedimented in 10 minutes at 800 ***g***, (b) material sedimented in 20 minutes at 4000 ***g***; (c) material sedimented in 60 minutes at 16,000 ***g***, and (d) "soluble" material, it is found that Fraction (b) has the highest specific activity. Furthermore, if freshly harvested cells are suspended in a buffered salt solution containing 10 per cent sucrose, disintegrated, fractionated in the same fashion, and the various fractions incubated with ^{14}C-glutamic acid or glycine, together with ATP as energy source, it is found that Fraction (b) is able to incorporate the labelled amino acid rapidly, having markedly greater activity than (a) while the activity of (c) and (d) is negligible. Electron microscope examination of

Fraction (b) shows that it consists of disrupted cells with a torn outer wall and a greatly decreased content of electron-dense material. This fraction constitutes the "disrupted cell preparation" used for the work to be described.

Properties of the disrupted cells

The disrupted cell is non-viable and possesses no measurable respiration but retains many of the enzymic and synthetic activities of the intact cell. It can metabolize glucose with the production of acid and can utilize hexosediphosphate as a source of energy for protein synthesis. If provided with an energy source and a complete mixture of the naturally occurring amino acids, it can synthesize protein as shown by increase in protein-nitrogen, in catalase and in glucozymase activity; if galactose is also supplied, as inducer, it can synthesize β-galactosidase although this enzyme is completely absent in the initial preparation. If provided with a mixture of purines and pyrimidines, including ^{14}C-uracil, and a source of energy, synthesis of ribonucleic acid (RNA) can be demonstrated by incorporation of radioactivity into the RNA fraction of the preparation, and this synthesis is increased by the further addition of a complete mixture of amino acids. As shown in earlier work with intact cells (Gale and Folkes, 1953*b*), the synthesis of RNA is markedly increased by the presence of chloramphenicol at a concentration which limits protein synthesis.

When disrupted cells are incubated with a ^{14}C-labelled amino acid and a source of energy (normally ATP + hexosediphosphate), the labelled amino acid becomes incorporated into the protein of the preparation. If the labelled amino acid is one component of a complete mixture of amino acids (condition 2), incorporation proceeds linearly for some hours and is accompanied by measurable increase in protein. If the labelled amino acid is the only amino acid present in the incubation mixture (condition 1), then the rate of incorporation, which may initially be greater than that occurring in the

presence of the complete mixture of amino acids, decreases with time and incorporation eventually ceases; the amount of amino acid incorporated when the process ceases corresponds to only a small proportion of the corresponding residues initially present in the protein fraction. In condition 1, no change in the protein content of the preparation can be demonstrated and it has been shown elsewhere (Gale and Folkes, 1955*a*; Gale, 1956*a* and *b*) that such incorporation cannot be taken as a measure of protein synthesis or as an indication that such synthesis is occurring unless supporting evidence is forthcoming. As a working hypothesis, it has been suggested that incorporation under condition 1 takes place as a result of an exchange reaction between amino acid added to the medium and corresponding residues in certain of the proteins present in the preparation. It may be that such exchange incorporation is an activity of a part or parts of the protein-synthesizing mechanism and that this activity can occur when total protein synthesis is not possible.

Effect of Nucleic Acid Removal

Nucleic acid can be removed from the disrupted cells by extraction with M-NaCl or incubation with ribo- or deoxyribonuclease. After such treatment the preparation is no longer able to synthesize protein unless the incubation mixture is supplemented by appropriate mixtures of nucleic acids or their precursors (Gale and Folkes, 1955*b*). The treatment also results in a decrease in incorporation of amino acids under condition 1; the degree of decrease varies with the amount of nucleic acid removed and also with the particular amino acid whose incorporation is being studied. The incorporation of glycine is particularly sensitive; removal of nucleic acid to the point where the content is less than 10 per cent of that in the initial cell results in reduction of glycine incorporation to 10–15 per cent of that which takes place in the intact cell.

Incorporation in nucleic acid-depleted preparations can be restored by addition of staphylococcal nucleic acid to the

incubation mixture. The action of added nucleic acid is to increase both the rate of incorporation and the amount of amino acid incorporated by the time the process ceases. Restoration can be brought about by the presence of either DNA or RNA but the latter is always less effective than the former; optimal concentrations of RNA normally restore incorporation to 50–70 per cent of the value obtained in the presence of optimal concentrations of DNA. Of a variety of nucleic acids tested in the staphylococcal system, only those obtained from *Staph. aureus* proved to be effective.

Modification of Nucleic Acid Component

Whatever may be the mechanism of incorporation under condition 1, it provides an experimental system in which a relationship between proteins, amino acids and nucleic acids can be investigated.

Effect of digestion of nucleic acid. If staphylococcal DNA is digested with deoxyribonuclease, the activity of the digest in restoring glycine incorporation is less than that of the intact nucleic acid and is further reduced on dialysis. If staphylococcal RNA is digested with ribonuclease, the effect of the digest in restoring glycine incorporation is greater than that of the undigested RNA, the digest promoting incorporation to the same level as that obtained in the presence of optimal concentrations of DNA. Whereas the restoration by intact RNA appears to be species specific, ribonuclease digests of RNA from a variety of sources prove to be effective and the active material in all cases is dialysable.

Attempts have been made to fractionate RNA digests, and activity in promoting incorporation has been found associated with two types of fraction. When fractionation is carried out according to the methods described by Markham and Smith (1952) for the separation of small polynucleotides, activity towards specific amino acids is found associated with specific polynucleotide fractions, whereas activating substances of a relatively non-specific nature ("incorporation

factors") are found in fractions which, from their physicochemical properties, are not nucleotides. It may be that specific polynucleotides are activated by substances related to the "incorporation factors" but definite information on this point awaits the characterization of the latter factors.

Effect of X-irradiation on the nucleic acid response. Table I shows that exposure to 150,000 r has no significant effect

Table I

Restoration of Glycine Incorporation by Staphylococcal Nucleic Acids Before and after X-irradiation

	Increase in glycine incorporation (c.p.m./mg.)		
Nucleic acid added: *mg./ml.*	0·1	0·2	0·2
		Deoxyribonucleic acid	
X-radiation dose (r)			
None	154	260	430
50,000	179	265	372
150,000	180	287	347
		Ribonucleic acid	
None	75	158	197
50,000	74	—	141
150,000	70	142	158

Incorporation in absence of added nucleic acid = 218

on the ability of staphylococcal RNA or DNA to promote glycine incorporation. These results provide a further indication that the ability to promote incorporation under condition 1 resides in portions of the nucleic acid structure rather than in the complete polynucleotide.

Inhibitors

Antibiotics. Chloramphenicol inhibits the incorporation of all amino acids so far tested; in no case does the inhibition reach completion. In all cases investigated, inhibition increases with increasing concentration of antibiotic but reaches a plateau value which varies with the amino acid whose incorporation is studied (see Table II). The synthesis of protein, whether measured by increase in protein-N, catalase or β-galactosidase,

is markedly more sensitive to chloramphenicol than any incorporation reaction and, moreover, inhibition of protein synthesis proceeds to completion.

Table II shows that penicillin and bacitracin inhibit the incorporation of certain amino acids and that the inhibition resembles that by chloramphenicol in reaching a different

Table II

INHIBITION OF AMINO ACID INCORPORATION (CONDITION 1) BY ANTIBIOTICS

	% *Inhibition by*						
	Chloramphenicol			*Penicillin*			*Bacitracin*
	3	30	300	0·03	0·3	30	100
Amino acid	μg./ml.	μg./ml.	μg./ml.	μg./.ml.	μg./ml.	μg./ml.	μg./ml.
Glutamic acid	18	60	63	25	47	53	63
Glycine	18	38	40	8	22	37	26
Alanine	20	22	22	6	12	15	20
Aspartic acid	20	79	80	2	10	11	18
Leucine	20	80	88	0	4	10	17
Threonine			33	0	2	15	15
Phenylalanine			60	0	5	11	0
Lysine	18	30	33	0	2	4	0
Arginine			35	0	0	0	0
Tyrosine			57	0	0	0	0
Proline			70	0	0	0	0
Valine			70	0	0	0	0

plateau level in each case but differs in that not all incorporation reactions are affected. The inhibitions by the three antibiotics are significantly the same whether incorporation is promoted by RNA or DNA, consequently the plateau effects are not due to differentiation between the nucleic acids.

Chelating agents. Attention has been paid to the possibility that metal activation is involved in the promotion of incorporation by nucleic acids. A preliminary survey of the action of chelating agents indicated that glycine incorporation was highly sensitive to inhibition by 8-hydroxyquinoline. No significant inhibition was obtained with 0·01 M-versene or α:α-dipyridyl.

8-Hydroxyquinoline. The inhibition by 8-hydroxyquinoline (oxine) is unusual in that 10^{-5} M-oxine produces 80–90 per

cent inhibition whereas 10^{-3} M is markedly less inhibitory. Fig. 1 shows the effect of oxine on glycine incorporation in nucleic acid-depleted cells incubated in the presence and absence of staphylococcal DNA; 10^{-5} M-oxine prevents the

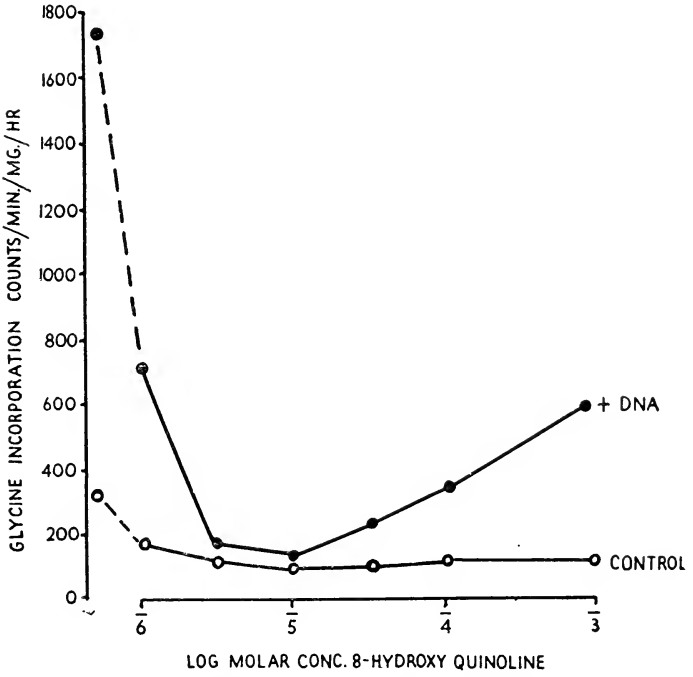


FIG. 1. Effect of 8-hydroxyquinoline on the incorporation of glycine in disrupted staphylococcal cells depleted of nucleic acid and incubated in the presence and absence of added staphylococcal deoxyribonucleic acid.

stimulation of incorporation by DNA. The effect is not specific for DNA since stimulation by RNA or by purified preparations of the "glycine incorporation factor" is abolished in the same way and at the same concentration. Albert, Gibson and Rubbo (1953) found that the bactericidal action of oxine depends upon the presence of heavy metal ions in the medium and that toxicity was maximal when equimolar quantities of

oxine and heavy metal were present. A similar situation appears to hold in the present instance since oxine is found to have no inhibitory action on glycine incorporation if the reagent solutions are "stripped" of heavy metals by the procedure of Waring and Werkman (1942) prior to test.

Metal effects. Glycine incorporation is highly sensitive to inhibition by copper ions and the toxicity of copper is greater in the absence of other metals such as iron or cobalt. In "stripped" incubation mixtures, glycine incorporation is 80 per cent inhibited by 10^{-6} M Cu^{++} while the promotion of incorporation by added DNA is abolished by concentrations of 10^{-7} to 10^{-6} M Cu^{++}. Although there is as yet no direct evidence for the participation of metals in amino acid incorporation, it may be that copper ions or oxine complexes of heavy metals block sites of reaction between nucleic acids and proteins and so prevent incorporation.

REFERENCES

ALBERT, A., GIBSON, M. I., and RUBBO, S. D. (1953). *Brit. J. exp. Path.*, **34**, 119.

ALLFREY, V. G. (1954). *Proc. nat. Acad. Sci., Wash.*, **40**, 881.

BELJANSKI, M. (1954). *Biochim. biophys. acta*, **15**, 425.

BORSOOK, H. (1954). Chemical Pathways of Metabolism. New York: Academic Press.

GALE, E. F. (1956*a*). *Symp. Biochem. Soc.*, in press.

GALE, E. F. (1956*b*). *Harvey Lectures*, in press. Springfield, Ill.: Charles C. Thomas.

GALE, E. F., and FOLKES, J. P. (1953*a*). *Biochem. J.*, **55**, xi.

GALE, E. F., and FOLKES, J. P. (1953*b*). *Biochem. J.*, **55**, 721, 730.

GALE, E. F., and FOLKES, J. P. (1955*a*). *Biochem. J.*, **59**, 661.

GALE, E. F., and FOLKES, J. P. (1955*b*). *Biochem. J.*, **59**, 675.

LESTER, R. L. (1953). *J. Amer. chem. Soc.*, **75**, 5448.

LITTLEFIELD, J. W., KELLER, E. B., GROSS, J., and ZAMECNIK, P. C. (1955). *J. biol. Chem.*, **217**, 111.

MARKHAM, R., and SMITH, J. D. (1952). *Biochem. J.*, **52**, 558.

NISMAN, B., HIRSCH, M. L., and MARMUR, J. (1955). *C.R. Acad. Sci., Paris*, **240**, 1939.

NISMAN, B., HIRSCH, M. L., MARMUR, J., and COUSIN, D. (1955). *C.R. Acad. Sci., Paris*, **241**, 1349.

SANGER, F. (1945). *Biochem. J.*, **39**, 507.

WARING, W. S., and WERKMAN, C. H. (1942). *Arch. Biochem.*, **1**, 303.

WEBSTER, G. C., and JOHNSON, M. P. (1955). *J. biol. Chem.*, **217**, 641.

ZAMECNIK, P. C., and KELLER, E. B. (1954). *J. biol. Chem.*, **209**, 337.

DISCUSSION

Spiegelman: I wonder whether staphylococcal RNAse can break down non-homologous RNA to active fragments. This could explain the specificity of the intact RNA, i.e. the RNA is actually broken down in those cases where it exhibits activity. Were this the case the specificity would react with the enzyme, not the RNA.

Gale: I cannot contradict that suggestion. I can only say that there is no detectable RNAse activity in the disrupted cell preparation.

Butler: I should like to mention results of some experiments by my colleague Dr. Hunter, which were actually begun in Dr. Gale's laboratory. He found an inhibiting effect of some nitrogen mustards on the incorporation phenomenon. This was with the intact bacterium under starved conditions. He has got very good correlation between the inhibition of incorporation and the inhibitory effect of these compounds on the Walker tumour. But if the examination is done under protein synthesis conditions there is no great inhibition, it is only found under the cell exchange conditions.

Pirie: What enzyme was it whose formation was inhibited by radiation of the DNA preparation, and did you get inhibition with any lower doses? Did you find any physical changes in your preparation of DNA after radiation with these doses?

Gale: The enzyme was β-galactosidase. I have not tested any other enzyme system. I have concerned myself principally with amino acid incorporation, where irradiation of the staphylococcal nucleic acids has no effect on their ability to promote glycine incorporation.

Cohn: This might be an appropriate time to make a few comments on nucleic acids, and in making them I imply no criticism of any particular work here or otherwise, but rather in the light of more exact interpretation of evidence which has been accumulated. There is no doubt that nucleic acids and the deoxyribonucleic acids, polydiesters of sugars and phosphates, do exist in tissue, and that they also exist in the preparations that are made from tissues. But it is a long step from there to assuming that the preparations that have been made (and this applies to all the preparations of which I have any knowledge) are anywhere near as clean as the letters or the formulae used to represent them. There is a great deal of doubt as to whether even crystalline proteins are 100 per cent what they are reputed to be, and certainly with respect to such a characterization as crystallinity the nucleic acid field is far removed. I think it was Gulland who said that "Nucleic acids are not compounds, they are methods of preparation". Now we know that there are impurities in nucleic acid preparations, and no one has reported much better than 90 per cent purity by any reliable means on any nucleic acid preparation. If we overlook the possible biological significance of 5 or 10 per cent, we overlook all that we know about trace elements and trace compounds such as enzymes. Furthermore, it is exceedingly difficult to remove ribonuclease or ribonuclease-like enzymes from nucleic acid preparations. Many nucleic acid preparations will autolyse themselves if given a chance, showing symptoms of contamination with their own

specific nucleases, whatever they may be. The heterogeneity, in many different ways, of nucleic acids is rapidly becoming apparent; heterogeneity according to site, as Prof. Davidson could attest; to metabolic activity in a specific site; to size. I am reminded of an observation coming from Dr. Markham's laboratory with respect to 5′ ends in nucleic acids and particularly in tobacco mosaic virus. The evidence of these 5′ ends depends on how you precipitate the virus. If you do it one way you get evidence of 5′ ends, if you do it another way you do not. Here is an apparent heterogeneity in terms of size or structure or admixed material which appears to depend on the method of precipitating the virus from the plant extract. Dr. Gale's evidence indicates that something (probably non-nucleotide as he himself says) which is carried by nucleic acids has a pronounced effect, an effect in which we are all interested. But in interpreting these effects we must remember that in a preparation of RNA and DNA the major component may indeed be what the letters stand for, but there is no guarantee that that is all that is present in the preparation.

Brachet: Dr. Gale said that, as a rule, intact cells are not permeable to nucleic acids. I think that the nucleic acids, at any rate when they are not very highly polymerized, can get into certain cells. They certainly can get into the amoebae. RNA can also get into the cells of the onion root: cytological evidence shows that it first produces extensive mitotic activity, followed by an inhibition. Most people still believe, and I believed up to two years ago, that a large molecule like a nucleic acid would not penetrate into a cell. I now think that we can no longer accept that this is always true.

With regard to the question of the possible rôle of copper, has copper any effect on RNA and protein metabolism, producing for instance a dissociation of the two? It has been found that there may be an accumulation of RNA in bacteria treated with cobalt under conditions where growth stops; I wonder whether copper can produce such an effect.

Gale: A dissociation of protein synthesis and nucleic acid synthesis has been shown with cobalt, but not, as far as I know, with copper. In the glycine incorporation system cobalt will antagonize the toxic action of copper, but whether or not this is due to differences in the affinity of their chelating systems, I don't know.

PROTEIN SYNTHESIS IN PROTOPLASTS*

S. Spiegelman
Department of Bacteriology, University of Illinois

Introduction

Weibull (1953) observed that exposure of *Bacillus megaterium* cells to lysozyme under hypertonic conditions leads to the formation of structures he labelled as protoplasts. Usually each rod-like cell yields two or three of the spherical protoplasts. Osmotically stabilized suspensions of the protoplasts were metabolically active, possessing a high endogenous respiration (Weibull, 1953) and capable of glucose oxidation at constant rates for extended periods of time (Wiame, Storck and Vanderwinckel, 1955). It was quite generally recognized that a subcellular system had been uncovered which could be potentially useful in the analysis of cell function. A number of laboratories immediately undertook a study of the synthetic capacity of protoplasts. It is the purpose of the present paper to summarize the results obtained to date.

The Synthetic Potentiality of Protoplasts

While we shall be mainly concerned with the synthesis of specific proteins, it is of interest to begin with studies demonstrating that protoplasts can support rather involved and extensive synthetic processes. It was shown independently in two laboratories (Brenner and Stent, 1955; Salton and McQuillen, 1955) that bacteriophage multiplication occurs in protoplasts of *B. megaterium* if the bacteria are infected or induced prior to the removal of the cell wall. Virus yields

* The original investigations described stemming from the author's laboratory were aided by grants from the National Cancer Institute of the U.S. Public Health Service and from the Office of Naval Research.

were in the neighbourhood of 30 per cent of those obtainable with intact cells. Single burst experiments demonstrated that virus synthesis was occurring in a major proportion of the infected protoplasts.

That protoplasts do indeed retain a major proportion of the synthetic potentiality of the cells from which they are derived is dramatically exhibited by Salton's (1955) experiments on spores. A modification of Hardwick and Foster's (1952) procedure for "committing" cells to sporogenesis was employed. The cells were then converted to protoplasts with lysozyme and incubated further. Approximately 1 per cent of the protoplasts were thereby converted to viable spores detectable by suitable plating procedures. Thus far no one has achieved direct conversion of protoplasts to viable cells by resynthesis of the cell wall. McQuillen (1955*c*) has, however, provided evidence indicating that protoplasts are capable of limited division. When properly supplemented, and incubated with aeration for periods extending between four and six hours, protoplasts take on dumb-bell shapes which are highly suggestive of the occurrence of incipient division.

Incorporation Studies

Attempts to study protein synthesis by tracer methods were actually made prior to the appearance of Weibull's publication and when it was not realized that under certain conditions treatment of sensitive cells with lysozyme results in the appearance of microscopically visible structural elements. Lester (1953) exposed *Micrococcus lysodeikticus* to lysozyme in the presence of sucrose and found that such "lysates" could still incorporate ^{14}C-labelled leucine into the protein fraction. The addition of deoxyribonuclease enhanced the incorporation, whereas ribonuclease abolished it. Similar findings were reported by Beljanski (1954), who used labelled glycine. Here again stimulation with DNAse and inhibition with RNAse were observed. It seems probable that both of these investigators were dealing wholly or in part with

protoplasts. We shall return subsequently to the significance of the results with the two nucleolytic enzymes.

The first extensive investigation of incorporation in defined protoplast preparations was performed by McQuillen (1955*a*). A variety of ^{14}C-labelled compounds was used, and a comparison of intact cells and protoplasts was made. The results obtained in the two were qualitatively similar. Thus, ^{14}C-carboxyl-labelled glycine made its way into the protein glycine and also into the adenine and guanine of the nucleic acids. The rate of incorporation in protoplasts was between 50 and 100 per cent of that observed in intact cells.

About the only striking difference between cells and protoplasts which emerged in these studies was a curious and unexplained dissimilarity in response to uranyl chloride. It was found that UO_2Cl_2 suppressed the incorporation of glycine into the nucleic acids of protoplasts but had no effect on the metabolism of intact cells.

Induced Synthesis of Protein

The induced synthesis of enzymes in suspensions of protoplasts was simultaneously achieved in three laboratories. Wiame and his collaborators (1955) showed that arabinokinase was formed in protoplasts prepared from *Bacillus subtilis* when they were incubated aerobically in the presence of arabinose, $(NH_4)_2SO_4$, yeast extract, and NaCl at 0·5M as a stabilizing agent. McQuillen (1955*b*, 1956) and Landman and Spiegelman (1955) demonstrated that protoplasts of *B. megaterium* strain KM can be induced to synthesize a β-galactosidase. I should now like to summarize the principal properties of this latter system. Unfortunately, McQuillen's findings have not as yet appeared *in extenso* and so comparison of the data obtained in the two laboratories is impossible.

Since they have already been detailed elsewhere (Landman and Spiegelman, 1955), we need not entertain here an extensive description of the conditions and stabilizing medium which were found to permit β-galactosidase formation in

protoplasts of *B. megaterium*. We may note, however, that, in addition to inducer, a supply of amino acids, hexosediphosphate, and aerobiosis were found to be essential. As with other synthetic functions, the properties of enzyme formation in protoplasts and intact cells were remarkably similar, providing the comparisons were carried out under hypertonic conditions.

The interest in the protoplast as a possible tool in the further analysis of enzyme synthesis stems essentially from the possibility that it would be more amenable to specific enzymatic resolution than the intact cell from which it is derived. Fortunately, this possibility is potentially attainable, for it is when one examines responses to various enzymes that striking differences between protoplasts and cells begin to emerge. This, for example, is clearly exhibited in Table I in the case

Table I

EFFECT OF TRYPSIN AND LIPASE ON ENZYME FORMATION IN CELLS AND PROTOPLASTS

Cells or protoplasts were suspended in inducer-free induction medium (0·5M-K_2HPO_4 at pH 7·8; 2 % amino acids, 0·6% hexosediphosphate) and incubated with the indicated enzyme for 1 hour, subsequent to which inducer (0·06 M lactose, final concentration) was added. The enzyme formed in the next two hours is recorded in terms of the mμM of *o*-nitrophenyl-β-D-galactoside hydrolysed per ml. per minute.

Enzyme Present	*Cells*	*Protoplasts*
None	1,090	620
Trypsin (100 μg./ml.)	1,140	0
Lipase (100 μg./ml.)	1,020	0

of the response to lipase and trypsin. Intact cells are completely insensitive to the enzymes whereas the synthetic ability of protoplasts is completely abolished.

These results illustrate a point worthy of the attention of those concerned with performing and interpreting experiments with subcellular fractions. One might perhaps be led to conjecture that a lipid is a key component of the enzyme-forming mechanism, based simply on the observation recorded with lipase. However, the fact is that the loss of enzyme-synthesizing ability is a simple consequence of the

physical dissolution of the protoplasts. After incubation with either lipase or trypsin at the levels indicated, few protoplasts can be recovered. It is thus important in any given case to demonstrate that an inhibition of enzyme synthesis which is observed to follow a particular treatment is not the result of a generalized destruction. This caution is also relevant to experiments involving ribonuclease (RNAse) and deoxyribonuclease (DNAse). Lysis of protoplasts by RNAse has been observed by Brenner (1955) and in our own laboratory. To be interpretable, experiments of this nature must be accompanied by evidence that the enzyme treatment has resulted in the *selective* removal of the homologous compound.

An extensive examination has been made (Spiegelman and Li, unpublished) of the effects of both RNAse and DNAse on the synthesis of β-galactosidase in both intact cells and protoplasts. No inhibitions were observed with intact cells under any conditions of test. Striking effects were, however, obtained with protoplasts. A few words may be interposed here on the conditions necessary for consistent results. Cells for an experiment were customarily prepared by inoculation with 2 per cent peptone and incubation with shaking overnight at 30°C. By morning, the cultures were in stationary phase and were put through a "rejuvenation" prior to use. This consisted in diluting the cultures fivefold with fresh medium and reincubating until they had entered logarithmic growth as determined by periodic examination of the optical density. It was noted that extraordinary care had to be exercised in controlling the extent of this rejuvenation if protoplast preparations were to be obtained exhibiting uniform behaviour with respect to enzyme-forming ability and response to enzymatic resolution.

Investigation of the cells during the course of the rejuvenation revealed that our procedure had inadvertently led to extensive phasing of the culture. This finding made understandable the extreme precision with which the timing of the rejuvenation had to be carried out. It also made possible the preparation of protoplasts which responded homogeneously

to the action of enzymes. These observations may be related to the recently published experiments of Thomas (1955) who noted a periodic variation in permeability of *Pneumococci* to such large molecules as DNA and DNAse.

It was also found that consistent removal of RNA and DNA by the corresponding enzymes can be achieved only if treatment is instituted during the formation of the protoplasts. Once protoplasts have been formed and have been incubated for a while in the stabilizing medium, they become relatively impervious to enzymatic resolution. It may be noted that in addition they become more and more resistant to the disruptive effects of lipase, although never completely so. The procedure employed for resolution may be outlined as follows. The cells are suspended in the hypertonic medium containing all supplements necessary for synthesis including amino acids and HDP but lacking the inducer. Lysozyme is added at a level of 200 μg. per ml. to convert the cells into protoplasts. In addition, at the same time, the enzyme to be tested for ability to resolve the protoplasts is included. The incubation is carried out for a period of 45 minutes at 30°C with constant shaking, by which time the cells will have been converted completely into protoplasts. The protoplasts are then recovered by centrifugation. An aliquot is removed for test of enzyme-forming ability; the remainder is retained for chemical analysis. Residual capacity to synthesize enzyme is examined by suspending the treated protoplasts in hypertonic medium containing amino acids, HDP, and inducer. The resulting suspension is incubated on a roller-type device for 2–3 hours with periodic sampling for enzyme assay. Controls are always run in parallel.

Table II summarizes a typical series of experiments in which the effect of DNAse on protoplasts is examined in terms of the percentage removal of DNA, RNA, and the residual enzyme-forming capacity. It will be noted that in some cases the treatment with DNA has led to the removal of some RNA. The reasons for this are still under investigation. The results, in so far as enzyme-forming abilities are

concerned, are clear-cut. It is quite evident from the data summarized in Table II that considerable amounts of DNA can be removed, up to 99 per cent, without loss of enzyme-forming capacity. However, it will be noted that in those cases where 30 per cent or more of the RNA is lost, serious inhibitions of enzyme-forming ability resulted.

Table II

THE EFECT OF DNAse ON ENZYME SYNTHESIS AND DNA AND RNA CONTENT

DNAse (400 μg./ml.) was present in the experimental flasks during protoplast formation (45 minutes). Protoplasts were then recovered by centrifugation and washed. An aliquot was used for determination of DNA, RNA and protein. The extent of removal of each nucleic acid is determined in terms of ratio to protein in the protoplast pellet and comparison with untreated control. This corrects for loss due to lysis during treatment. Enzyme-forming ability is examined with another aliquot of the protoplasts which is resuspended in an induction mixture (0·5M-K_2HPO_4, pH 7·8; 2% amino acids, 0·6% hexose-diphosphate, and, 0·06M lactose). Samples are removed periodically for enzyme assay. Enzyme activity is determined in terms of the mμM of *o*-nitrophenyl-β-D-galactoside hydrolysed per mg. of protein per minute. The rate of enzyme formation is obtained as the number of enzyme activity units synthesized per mg. of protein per hour.

Experiment	*Percentage removal*		*Enzyme formed (in percentage of untreated controls)*
	DNA	*RNA*	
$1020C_1$	87	0	400
$1029C_2$	94	0	420
$1020C_3$	97	0	540
$1019D_2$	41	4	104
$1019C_2$	43	17	120
$1019C_3$	99	13	100
718	65	31	13
715	65	32	15
719	59	46	0
712	39	42	12

Table III gives a comparable series of experiments in which the protoplasts were treated with RNAse, and here the picture is also clear. In most cases, there is relatively little concomitant loss in DNA. Again, one observes that wherever the removal of RNA exceeds 35 per cent, drastic inhibitions of enzyme-forming capacity results.

The data obtained in the experiments just described support

Table III

THE EFFECT OF RNAse ON ENZYME SYNTHESIS AND DNA AND RNA CONTENT

RNAse (500 μg./ml.) was present in the experimental flasks during protoplast formation (45 minutes). Protoplasts were then recovered by centrifugation and washed. An aliquot was used for determination of DNA, RNA and protein. The extent of removal of each nucleic acid is determined in terms of ratio to protein in the protoplast pellet and comparison with untreated control. This corrects for loss due to lysis during treatment. Enzyme-forming ability is examined with another aliquot of the protoplasts which is resuspended in an induction mixture (0·5M-K_2HPO_4, pH 7·8; 2% amino acids, 0·6% hexosediphosphate, and 0·06M lactose). Samples are removed periodically for enzyme assay. Enzyme activity is determined in terms of the mμM of *o*- nitrophenyl-β-D-galactoside hydrolysed per mg. protein per minute. The rate of enzyme formation is obtained as the number of enzyme activity units synthesized per mg. protein per hour.

Experiment	*Percentage removal*		*Enzyme synthesized (in percentage of untreated controls)*
	DNA	*RNA*	
1013	21	33	30
930B	0	34	0
926B	0	36	21
1004D	14	39	38
1004C	16	52	14
1029B	0	54	16
1004F	0	72	10
1014B	0	72	0
1020B2	13	75	1
1020B3	58	78	0

the conclusions derived from the study of other subcellular systems, such as those developed by Gale and Folkes (1955*a* and *b*) and by Zamecnik and his collaborators (Zamecnik and Keller, 1954). They suggest that the molecular integrity of RNA is essential for the synthesis of new protein molecules.

REFERENCES

BELJANSKI, M. (1954). *Biochim. biophys. acta*, **15**, 425.
BRENNER, S. (1955). *Biochim. biophys. acta*, **18**, 531.
BRENNER, S., and STENT, G. S. (1955). *Biochim. biophys. acta*, **17**, 473.
GALE, E. F., and FOLKES, J. P. (1955*a*). *Biochem. J.*, **59**, 661.
GALE, E. F., and FOLKES, J. P. (1955*b*). *Biochem. J.*, **59**, 675.
HARDWICK, W. A., and FOSTER, J. W. (1952). *J. gen. Physiol.*, **35**, 907.

LANDMAN, O. E., and SPIEGELMAN, S. (1955). *Proc. nat. Acad. Sci., Wash.*, **41**, 698.
LESTER, R. L. (1953). *J. Amer. chem. Soc.*, **75**, 5448.
MCQUILLEN, K. (1955*a*). *Biochim. biophys. acta*, **17**, 382.
MCQUILLEN, K. (1955*b*). *J. gen. Microbiol.*, **13**, iv.
MCQUILLEN, K. (1955*c*). *Biochim. biophys. acta*, **18**, 458.
MCQUILLEN, K. (1956). *Symp. Soc. gen. Microbiol.*, in press.
SALTON, M. R. J. (1955). *J. gen. Microbiol.*, **13**, iv.
SALTON, M. R. J., and MCQUILLEN, K. (1955). *Biochim. biophys. acta*, **17**, 465.
THOMAS, R. (1955). *Biochim. biophys. acta*, **18**, 467.
WEIBULL, C. (1953). *J. Bact.*, **66**, 688.
WIAME, J. M., STORCK, R., and VANDERWINCKEL, E. (1955). *Biochim. biophys. acta*, **18**, 353.
ZAMECNIK, P. C., and KELLER, E. B. (1954). *J. biol. Chem.*, **209**, 337.

DISCUSSION

Davidson: There is one point I should like to ask in connection with technique. How, if you are working to a matter of minutes, can you know when to take your sample? By the time you have got your DNA estimation it is too late.

Spiegelman: We take our 10-minute samples blindly. The plateaus are 30 minutes long and sampling at 10-minute intervals is adequate to exhibit them.

Gale: Is there no change in the turbidity associated with the stepwise increase in the DNA?

Spiegelman: You do not see cycling of overall protein synthesis but there is apparent cycling of induced enzyme formation. There is a very interesting possibility here which I think fits in with the hypothesis proposed by Gale, namely that the RNA templates of the induced enzymes are unstable. They would therefore require recharging from the nucleus, and in this event formation of such proteins would be more closely tied to nuclear events than would constitutive protein formation. However, it is not possible at the present time to make any statement that such a difference is actually real. It should be noted that the absence of cycling in the constitutively formed proteins does not mean that none exist, because one can imagine that they are all cycling but are out of phase with each other; and so the effect is cancelled out. What one has to do is follow the synthesis of particular constitutive proteins. We have been looking for such, but we haven't as yet found any that possess all the properties that would make them suitable material for study.

Alexander: Does the actual numerical increase in bacteria also follow a plateau if you take them out?

Spiegelman: No, not in this system. That is difficult to do because this is multicellular; each cone is not a single cell, we have had to shake them apart to get good correspondence.

Alexander: Therefore it is possible that the actual number of cells may also go stepwise.

Lajtha: Does this cycling imply that the lag-phase which we usually see is only apparent, and that in the first few cycles the number of cells are so few that one does not see them? We were worried about this some time ago and started cultures with high numbers initially, and then we did not see any lag-phase at all. Furthermore, functionally these bacteria in the so-called lag-phase were sensitive to very small concentrations of nucleic acid analogues, and to very high concentrations, about 50 times that much, in the log-phase. If we started the culture with the same high numbers of bacteria that were present in the log-phase, we had to use again high concentrations of analogues to achieve comparable inhibition of growth.

Spiegelman: I don't think that all lag-phases are going to be explained on this basis. I do, however, think that this illustrates a rather interesting point. Many biologists have used an enormous amount of ingenuity with microbial populations to get phased cultures. In actual fact it may be difficult to avoid them. We just let the thing go to a stationary phase and start it off again and it is phased; but it does not stay phased very long. We obtain four good cycles and that is about all.

Gale: How long are your inoculum cells in the stationary phase before rejuvenation?

Spiegelman: About five hours.

Krebs: How do the protoplasts obtain energy? Do they respire?

Spiegelman: Yes, they do respire, but we give them HDP, which seems to be the thing they like the best.

Krebs: Am I wrong in assuming that this is one of the organisms that does not ferment anaerobically?

Spiegelman: It is essentially an aerobic organism.

Brachet: What do you know about the relationship between this cycling and the time of DNA synthesis? Is there any correlation between the stage where DNA is being synthesized and the stage where the enzyme is being synthesized?

Spiegelman: It is very difficult to get really accurate information on that, although we have discovered one thing which may help a lot. You can freeze this thing in whatever stage it is by simply raising the osmotic pressure. I don't know why this should work, but it does. It will freeze it for a matter of hours. Our data suggest that you don't get complete coincidence of the enzyme and DNA-synthesizing plateaus.

Brachet: Have you studied further the stimulating effect of the removal of DNA? It is of course rather reminiscent of what happens in *Acetabularia* after removal of the nucleus.

Spiegelman: It is tempting to imagine that the removal of the DNA actually decreases the ability of the preparation to synthesize certain proteins. This may give added advantage to the one which is being induced, since the inducer is present, and consequently leads to an increase in its formation. I should like to emphasize that the experiments I have described are not as decisive as yours, because when I say I remove the DNA all I can mean is that I remove the DNA as measured

by a chemical operation in which all the soluble components are extracted with cold perchloric acid. I should like to ask how big a piece of DNA would be precipitated by, say, cold 0·2 N-perchloric acid.

Cohn: I should say from four nucleotides onwards you are running into danger of such precipitation; and since DNAse does leave pieces of four, five and six nucleotides, there could be DNA polynucleotide in such a precipitate.

Spiegelman: In that case, my conclusion is strengthened. The data reported are based on the chemical analysis of the precipitate. It would suggest that I don't have anything larger than four nucleotides. It would appear then that I must have broken the DNA down into very small pieces.

Cohn: Yes, if there is nothing in the precipitate.

Spiegelman: We do look in the precipitate. We give an enormous dose of DNAse, in order to get the treatment through in time. It should degrade very fast if it is going to go at all.

INFLUENCE OF RADIATION ON DNA METABOLISM

ALMA HOWARD

British Empire Cancer Campaign Research Unit in Radiobiology, Mount Vernon Hospital, Northwood

SINCE the discovery by Hevesy and his colleagues (for references, see Hevesy, 1948) that the incorporation of ^{32}P into DNA of Jensen sarcoma and other tissues of the rat was markedly reduced by X-irradiation, it has been recognized that interference with DNA synthesis is one of the most general and important biological effects of radiation. It has been generally supposed that ionizing radiation interrupts the DNA biosynthetic chain by altering some reaction along its course, and it was natural therefore to examine the possibility that some step or steps in the biosynthesis would prove to be especially radiosensitive, and that the effects of irradiation might be altered by manipulation of the metabolites involved in such steps. Hevesy (1949) showed that the uptake of ^{14}C-acetate into DNA of rat tissues was depressed by X-rays, as was that of ^{32}P. A number of papers since have indicated that a large part, perhaps the whole, of the biosynthetic process is affected by radiation. It has been claimed, however, that the incorporation of labelled adenine is not reduced by doses that have a marked effect on the entry of formate, glycine or orotic acid into DNA. This claim has been made from two sources:

(1) Harrington and Lavik (1955) found that the incorporation of [8-^{14}C]adenine into DNA purines of rat thymus, during the period 30 minutes to 24 hours after 100 r whole body X-rays, was significantly greater than in controls. In the same experiment the incorporation of [2-^{14}C]orotic acid into DNA pyrimidines and of ^{14}C-formate into purines was

depressed. Bennett and Krueckel (1955) have repeated this experiment and observed a marked depressing effect of irradiation on the incorporation of [8-^{14}C]adenine, the DNA specific activity being less than half that of controls. No explanation is apparent for the discrepancy between this result and that of Harrington and Lavik.

(2) Passonneau and Totter (1955) found no inhibition of incorporation of [8-^{14}C]adenine into purines of DNA in chick embryos *in vitro*. Doses were from 1,000 to 20,000 r of gamma-rays. The incorporation of ^{14}C-formate and ^{14}C-glycine was reduced after doses of 5,000 r or more: 1,000 r had a variable effect. The chick embryos, which were suspended in saline for the two-hour period of the experiment, showed a rate of DNA labelling with formate and glycine that was probably less than that expected from the increase in amount of DNA *in vivo* in the same period. Embryos whose hearts had stopped had the same performance as survivors of the 20,000 r dose. Very large doses were required to have any clear effect on DNA metabolism, although Lavik and Buckaloo (1954) found an approximately 50 per cent inhibition of ^{14}C-formate or ^{14}C-cytidine uptake into DNA of chick embryos after 400–450 r. These facts suggest that this experimental system cannot be regarded as showing normal biosynthesis of DNA. If they are accepted as doing so, it could be argued that adenine is incorporated by exchange, since Brown (1950) found that the renewal rates for both RNA and DNA were higher with adenine than with ^{32}P or ^{15}N-glycine. (Payne, Kelly and Jones (1952), however, did not observe a higher incorporation with [4:6-^{14}C]adenine than with ^{14}C-formate, [2-^{14}C]glycine, or ^{32}P).

Taking all the evidence together, it appears that there is no very clear indication that irradiation interferes with any particular step in DNA synthesis. The blockage seems rather to be a general one. This suggests that the inhibition of DNA synthesis may not be a primary effect of radiation, but is the result of blockage in some other event in the development of the cell.

Effect of irradiation on DNA metabolism in some mammalian tissues

An examination of the literature shows that very wide differences exist between tissues in their response to irradiation with regard to its effect on DNA metabolism. This is especially clearly shown in the work of Kelly and co-workers (1955), who measured the incorporation of ^{32}P into DNA of mouse small intestine, spleen, liver, bone marrow ("carcass"), and two transplanted tumours after X-irradiation at four dose-levels. Informative time curves were obtained by sacrificing animals from 2 hours to 5 days after irradiation, the isotope being injected at fixed short times before sacrifice. The responses of the tissues were so different, both in dose and time-response, that no general statement can be made about them other than that a depression was always seen, and that it was apparent at the earliest times after irradiation that were studied. In the small intestine, for example, the maximal effect of 800 r was seen at 3 hours, with full recovery at about 1 day and nearly three times the normal rate of incorporation at 2 days. Bone marrow incorporation, on the other hand, showed a maximal effect only at about 3 days after 800 r, and was still very low at 5 days. The different response of the two tumours, a mammary carcinoma and a lymphosarcoma, was especially striking. They had approximately the same growth-rate, mitotic index, and short-term incorporation of ^{32}P before irradiation. After 800 r the mammary carcinoma showed a reduction in incorporation to about one half, but no change in weight or histological appearance beyond a decrease in mitotic index. The lymphosarcoma showed reduction in incorporation to 4 per cent of normal at one day, with later apparent recovery; there was marked involution, and a large amount of cell death. It is very clear that there had been important changes in the cell population, and the authors point out that most of the effects they observed in this and other tissues could be explained by such changes. The reduction in amount of various phosphorus compounds, including DNA, in rabbit bone marrow after gamma-ray doses in the mean

lethal range has been related to alterations in the number and type of cells present (Thomson *et al.*, 1953). The instances cited are only two of numerous experiments in which, as well as the changes in DNA metabolism that were being studied, alterations in the numbers and types of cells in the tissue were clearly being produced by the irradiation at the same time.

Radiosensitivity of DNA metabolism

In some tissues (Holmes and Mee, 1955; Harrington and Lavik, 1955) the effect of 100 to 150 r in reducing DNA turnover can be clearly seen. While these doses are much lower than those required to alter measurably many other processes of cell metabolism, they are well above the minimum for causing delays in the mitotic cycle. It is natural to ask whether two so very radiosensitive effects may not be causally related, and Hevesy many years ago suggested that cells may be delayed by irradiation in entering mitosis because they have been prevented from synthesizing their normal amount of DNA. In several tissues, however, the synthesis of DNA appears to be completed some time before prophase begins (Howard and Pelc, 1953; Lajtha, Oliver and Ellis, 1954) and it is not easy to see how stoppage of DNA synthesis can be directly responsible for delay in cells already on the brink of prophase, and containing their full double quantity of DNA. There seems no doubt that these cells are sensitive to delay since, in a great many tissues that have been studied, irradiation is followed very quickly by a fall in the number of cells entering mitosis.

Hevesy also pointed out that the delay in mitosis caused by irradiation would result in interference in DNA synthesis. In 1945 he said: "Since the ionizing radiation blocks cell division, it will influence the said cycle of changes [DNA synthesis], and a reduction in the number of desoxyribose nucleic acids built up during a given period of time can be expected to take place." It seems important to examine the implications of this statement in the light of knowledge of DNA metabolism that has accumulated over the past ten

years. It is now clear that synthesis of DNA is a function of the mitotic cycle. This is inherent in the fact that in any given species, a fixed amount of DNA is associated with each chromosome set, so that, with due allowance for differences in ploidy and for periods of synthesis, each nucleus contains a constant amount of DNA. This means that each cell must double, but no more than double, its content of chromosomal DNA during every interphase that is to be followed by a mitosis. Furthermore, the time period in interphase occupied by this synthesis appears to be fixed for any given cell type. As far as we know, no other component of the cell behaves in this fashion as regards amount per cell or dependence on the mitotic cycle, so that DNA synthesis might be expected to be unique in its response to radiation-induced changes in that cycle.

Radiation-Induced Changes in Cell Populations

The changes in the cell population that result from irradiation of growing tissues arise in the following ways:

(1) Delay in entry of cells into and progress through mitosis, expressing itself as a shift in the proportion of cells in various stages of the mitotic cycle. Larger doses cause longer delays. The sensitive period for delay is just before visible prophase (in the grasshopper neuroblast, during prophase). Recovery after moderate doses is characterized by a temporary increase in the number of cells in mitosis due to the release of those delayed. In the most favourable material, the delaying effect of 4 r can be observed (Carlson, 1948). In many other tissues, delays are known to result from very moderate doses. In some such cases, protein synthesis, RNA turnover, and increase in cell volume and dry weight all appear to be unaffected (Klein and Forssberg, 1954).

(2) Death of cells.

(a) Due to physiological or morphological changes in the chromosomes. Such changes may result in death of cells at metaphase or anaphase of the mitosis following irradiation or later, usually during the following interphase, due presumably to loss of genetic material. The rate at which cells die in this

manner depends on the rate at which they reach mitosis. The extent of tissue damage is greater at higher doses, dose rates, and ion density of the radiation.

(b) In a manner not known to be associated with physiological or morphological changes in the chromosomes, and not due to loss of genetic material. Cells may die upon attempting division (Laznitski, 1943*a*; Oakberg, 1955); in this case, the rate of cell death again depends on the rate at which cells reach mitosis, as in (a). They may, on the other hand, die during the interphase in which they were irradiated, independently of any recovery of mitotic activity. The sensitivity of cells to this kind of interphase death is enormously varied. Lymphocytes in the lymph nodes are rapidly destroyed by 100 r (Trowell, 1952): in mouse ascites tumour there is no evidence of cell death after 1250 r, at least until mitosis reappears (Klein and Forssberg, 1954); chick fibroblasts in culture exhibit interphase death at 2,500 r (Laznitski, 1943*b*); and some differentiated tissues having no measurable mitotic activity are histologically unaffected by even higher doses (Bloom, 1948*a*). In this respect, the lymphocyte appears to be very exceptional, and it seems reasonable to regard interphase death, of a kind unrelated to mitosis, as very unlikely in most tissues except after doses of well over 1,000 r.

Results of Changes in Cell Populations

After moderate doses of radiation, i.e. less than about 1,000 r, the shifts described would be expected to affect the amount of DNA being synthesized in a tissue as follows:

(1) The *time* at which the normal supply of cells entering synthesis is reduced will depend upon the time in the cell cycle at which DNA is normally synthesized. If synthesis begins immediately after telophase, irradiation will have an early effect in reducing the number of synthesizing cells. If there is a time lag between telophase and synthesis, the effect of irradiation will be deferred until this time has elapsed. Fig. 1 shows the time of uptake of labelled precursors into DNA, and the estimated lengths of other periods, in the mitotic cycles

of three tissues which have been studied by means of autoradiographs. In the bean root meristem, (Fig. 1 A), there is a G_1 period of up to 12 hours; the number of cells whose DNA becomes labelled with ^{32}P remains normal for at least 6 hours after irradiation (Howard and Pelc, 1953). In the Ehrlich

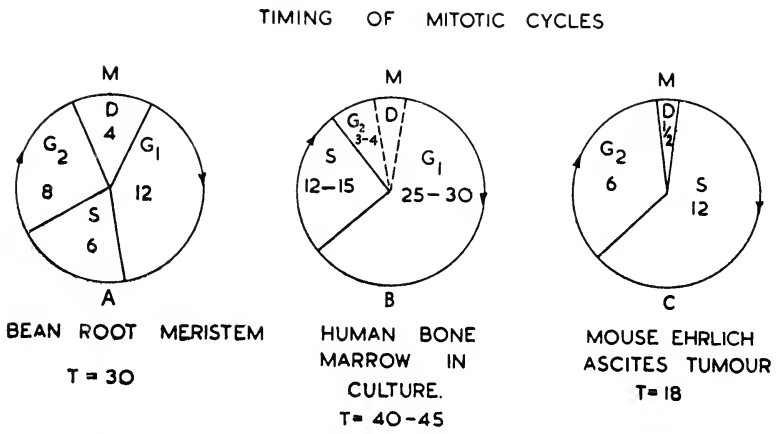


FIG. 1. Mitotic cycles deduced from autoradiograph studies. Time in hours. M = metaphase; D = mitotic division; S = period of uptake of isotope into DNA; G_1 and G_2 = periods in early and late interphase during which DNA does not become labelled; T = total length of mitotic cycle.

A. Bean root meristem. ^{32}P
(Howard and Pelc, 1953.)
B. Human bone marrow. ^{32}P or [8-^{14}C]adenine
(Lajtha, Oliver and Ellis, 1954.)
C. Mouse Ehrlich ascites tumour. [8-^{14}C]adenine
(Hornsey and Howard, 1956.)

mouse ascites tumour (Fig. 1 C), no G_1 is observed; within 2 hours after irradiation there is a decrease in the number of cells taking up [8-^{14}C]adenine into DNA (Hornsey and Howard, unpublished). If measured biochemically, the mitotic delay in the tumour would appear as a reduction in DNA turnover and specific activity, compared with controls, although the amount of DNA per cell would increase for a time equal to G_2 (see Fig. 1), and remain slightly greater than controls until mitosis reappeared. Such effects are not

incompatible with the published results of irradiation experiments in this material (Klein and Forssberg, 1954; Forssberg and Klein, 1954), and are in agreement with some recent work of Kelly (1955).

(2) The *degree* to which DNA synthesis is affected will be determined largely by the length of S (see Fig. 1) in relation to the time length of the premitotic block. Thus the synthesis of DNA in a tissue will be reduced to zero if the mitotic block (plus G_1 if it exists) is longer than S. In the Ehrlich ascites tumour, a dose of 400 r stops mitosis for 9 to 12 hours. Since S is about 12 hours, we would not expect to observe a period when there was no synthesis, and after 12 hours there would be a larger than normal number of synthesizing cells, as has in fact been observed (Hornsey and Howard, unpublished).

(3) The *recovery* of DNA synthesis in a tissue will depend upon the factors discussed under (2), i.e. the degree of depression, and also on the rate of cell death, the degree of tissue disturbance caused by it, and any other effect which the presence of dead or dying cells may have on the metabolism of the survivors. These last two points we know little about. The effect on specific activity will further depend on the rate at which dead cells are removed from the population, either by phagocytosis, migration, or some concomitant of differentiation. The rate of return of the tissue to normal will be influenced by its normal rate of cell replacement: thus in the small intestine, where epithelial cells normally have a lifetime of approximately 2 days (Leblond, Stevens and Bogoroch, 1948; Knowlton and Widner, 1950), the regeneration of the epithelium is very rapid (Bloom, 1948*b*) and DNA synthesis has recovered by 1 day (Kelly *et al.*, 1955). The mitotic index in the rat intestinal mucosa recovers by 3 days after 1,000 r (Webber, Craig and Friedman, 1951).

Conclusions

In view of these considerations, it is plain that a purely biochemical analysis of a growing tissue containing cells at all mitotic stages cannot tell us whether the inhibition of DNA

synthesis is due only to changes in the cell population, or whether we may infer that irradiation is also having a primary biochemical effect. For this information we must look to experiments of the following kinds:

(1) Biochemical analysis of growing tissues supplemented by studies on mitotic delay and cell death. The few published studies on material for which such information, however fragmentary, is at hand, are compatible with the view that inhibition of DNA synthesis is a result of radiation-induced changes in the mitotic cycle and in the cell population. On the other hand, it must be recognized that there are in the literature some experimental results which can be explained as due entirely to cell population changes only by assuming characteristics of the normal mitotic cycle that may appear unlikely. Thus Hevesy (1945) observed that in Jensen rat sarcoma, a dose between 335 and 1,500 r reduced ^{32}P uptake into DNA to less than half of normal within one hour of irradiation. Unless this is an interphase effect, one must suppose that synthesis follows telophase directly and occupies less than 2 hours.

(2) Observations on individual cells. This has been done with autoradiographs in experiments discussed previously. In the case of bean roots, Howard and Pelc (1953) concluded that the most probable length of G_1 was 12 hours, and therefore that DNA synthesis was inhibited in cells irradiated earlier in the same interphase, i.e. 6–12 hours before the beginning of synthesis. Since, however, the length of G_1 cannot be rigorously fixed from the information available, it is not excluded that the sensitive period for inhibition of DNA synthesis may coincide with that for delay in mitosis. The results of irradiating the ascites tumour (Hornsey and Howard, unpublished) are, as already stated, those to be expected from mitotic delay. Lajtha, Oliver and Ellis (1954) observed an immediate effect on synthesis of DNA in human bone marrow cells in culture. The dose used was, however, so large (5,000 r) that interphase death is to be suspected. No cell was observed to enter mitosis after this dose.

A second method of observing irradiation effects on DNA synthesis in single cells is that of photometric measurement of the amount of Feulgen stain. Grundmann's (1953) results on bean root meristems at 2 or 4 hours after 200 r, or 4 hours after 800 r, suggest that the changes in DNA classes could be explained by mitotic delay and cell death.

(3) Analysis of tissue which is synchronized with regard to the mitotic cycle. Such a situation is approached by mammalian liver regenerating after gross damage such as partial hepatectomy or CCl_4 poisoning. Since DNA synthesis begins before mitosis appears, its interruption by moderate doses of radiation cannot be due simply to mitotic inhibition. This tissue thus stands as one for which this simple hypothesis is definitely untenable. Since it is to be discussed in another paper at this meeting, no comments need be made here.

In conclusion, it appears that in regenerating liver there is some reason to think that DNA synthesis may be specifically interrupted by moderate doses of ionizing radiation. In other tissues, although it is possible that this is so, much more needs to be known about the changes in cell population that result from irradiation before a primary biochemical interference can be established with certainty. Meanwhile, we must recognize that most experimental results can be explained as due simply to mitotic delay and cell death, and do not require us to invoke a biochemical action of radiation on DNA synthesis *per se*.

REFERENCES

Bennett, E. L., and Krueckel, B. J. (1955). Univ. of Calif. Radn. Lab. Reports 2827–2828.

Bloom, W., ed. (1948*a*). Histopathology of Irradiation from External and Internal Sources, Chaps. 11, 18, 19. New York: McGraw Hill.

Bloom, W., ed. (1948*b*). Histopathology of Irradiation from External and Internal Sources, Chap. 10. New York: McGraw Hill.

Brown, G. B. (1950). *Fed. Proc.*, **9**, 517.

Carlson, J. G. (1948). *J. cell. comp. Physiol.*, **35**, Suppl. **1**, 89.

Forssberg, A., and Klein, G. (1954). *Exp. Cell. Res.*, 7, 480.

Grundmann, E. (1953). Zweites Freiburger Symp. über Grundlagen und Praxis chemischer Tumorbehandelung, p. 187.

HARRINGTON, H., and LAVIK, P. S. (1955). *Arch. Biochem. Biophys.* **54**, 6.
HEVESY, G. (1945). *Rev. Mod. Phys.*, **17**, 102.
HEVESY, G. (1948). Radioactive Indicators. New York and London: Interscience Publishers.
HEVESY, G. (1949). *Nature, Lond.*, **163**, 869.
HOLMES, B. E., and MEE, L. K. (1955). Radiobiology Symposium 1954. London: Butterworth.
HORNSEY, S., and HOWARD, A. (1956). *Ann. N.Y. Acad. Sci.*, **63**, 915.
HOWARD, A., and PELC, S. R. (1953). *Heredity*, **6**, Suppl., 261.
KELLY, L. S. (1955). Univ. of Calif. Radn. Lab. Report 3268, p. 50.
KELLY, L. S., HIRSCH, J. D., BEACH, G., and PAYNE, A. H. (1955). *Radiation Res.*, **2**, 490.
KLEIN, G., and FORSSBERG, A. (1954). *Exp. Cell. Res.*, **6**, 211.
KNOWLTON, N. P., and WIDNER, W. R. (1950). *Cancer Res.*, **10**, 59.
LAJTHA, L. G., OLIVER, R., and ELLIS, F. (1954). *Brit. J. Cancer*, **8**, 367.
LAVIK, P. S., and BUCKALOO, G. W. (1954). *Radiation Res.*, Abstracts, **1**, 221.
LAZNITSKI, I. (1943*a*). *Brit. J. Radiol.*, **16**, 61.
LAZNITSKI, I. (1943*b*). *Brit. J. Radiol.*, **16**, 138.
LEBLOND, C. P., STEVENS, G. E., and BOGOROCH, R. (1948). *Science*, **108**, 531.
OAKBERG, E. (1955). *Radiation Res.*, **2**, 369.
PASSONNEAU, J. V., and TOTTER, R. R. (1955). *Radiation Res.*, **3**, 304.
PAYNE, A. H., KELLY, L. S., and JONES, H. B. (1952). *Cancer Res.*, **12**, 666.
THOMSON, J. F., TOURTELLOTTE, W. W., CARTTAR, M. S., and STORER, J. B. (1953). *Arch. Biochem. Biophys.*, **42**, 185.
TROWELL, O. A. (1952). *J. Path. Bact.*, **64**, 687.
WEBBER, B., CRAIG, B. R., and FRIEDMAN, N. B. (1951). *Cancer*, **4**, 1250.

DISCUSSION

Hollaender: The work of Dr. Gaulden which Dr. Howard referred to was done on the grasshopper neuroblast. Dr. Gaulden has shown that one can counteract the effects of radiation on the rate of mitosis by placing the neuroblast in a hypertonic salt solution immediately after irradiation (see p. 303).

Van Bekkum: How long does the irradiation take?

Hollaender: It takes about 1 minute.

Alexander: Could you tell us the experimental details of this treatment with hypertonic salt?

Hollaender: It contains 1·2 times the concentrations of inorganic salts in the medium isotonic to the grasshopper neuroblast. In other words, it is only slightly hypertonic to the cells.

Alexander: Could you tell us the time of immersion in the more concentrated salt solution?

Hollaender: Throughout observations (264 minutes).

Spiegelman: Why was that tried?

Hollaender: There were several reasons: (1) Harrington and Koza (1951, *Biol. Bull.*, **101**, 138) working with grasshopper neuroblasts, found that the cells swelled almost immediately after treatment with 100 r or more of X-rays. This suggested a radiation-induced change in osmotic-pressure relationships in the cells. The cells looked as they would do had they been placed in medium hypotonic to them.

(2) The radiation-induced "reversion" of middle and late prophase neuroblast chromatin to an interphase condition, a primary cause of mitotic inhibition in this cell, resembles the "disappearance" of chromosomes produced when cells are placed in solutions hypotonic to them.

(3) Gaulden found that the chromatin of telophase, interphase and early and middle prophase cells could be made to resemble chromatin of late prophase by placing the cells in culture medium hypertonic to them. This change occurred within seconds and was accompanied by an accelerated mitotic rate (Gaulden, M. E. (1956), "Visible characteristics of living interphase and mitotic chromatin in the grasshopper neuroblast and the effects of abnormal toxicity on them." In manuscript).

These observations together with those of Sugiura (1937, *Radiology*, **29**, 352) who found growth capacity of irradiated tumour fragments to be increased when placed in hypertonic solutions, led Gaulden to test the efficacy of hypertonic medium in counteracting radiation-induced reversion of chromosomes, which results in mitotic inhibition.

Lajtha: Dr. Howard mentioned the possible interphase killing effect of 5000 r, which I think is a very important point. It undoubtedly kills some cells, you can see them dying in certain cultures. However, the numbers are relatively low. We repeated the experiments with 1000 r and I think we have indication for an interphase effect. The G_1 period is very long, or relatively long in the bone marrow cells, of the order of 20 hours or more. If, therefore, the cells would be damaged only during mitosis, then for a considerable time afterwards undamaged G_1 cells would enter and go through their synthetic period making the normal amount of DNA. We find on the other hand that even after 1000 r all the cells which enter the synthetic period produce only a fraction of the normal amount of DNA. The grain counts instead of the normal 60–80 are of the order of 10 or less, and we don't see any appreciable number of dying cells after 1000 r. This rather suggests that the whole G_1 period is damaged by radiation. The one difficulty is that 1000 r has a direct effect on the synthetic period as well, i.e. it will stop DNA synthesis then and there. I think we must repeat these experiments with 300 r or less, as you did with 150 r.

Howard: These effects ought to be separable since the dose effect is so widely separated. One can have a big mitotic delay with 100 r.

Lajtha: A dose of 150 r did not inhibit the synthetic period in the bone marrow cells, just as in your experiments with bean roots it did not inhibit the S period.

Swanson: May I ask your opinion about this first effect of radiation in terms of DNAse, i.e. where the chromosomes become sticky; is there any clue as to what is actually happening there?

Howard: It has been proposed that this was due to the depolymerization of DNA on the chromosomes. I don't think there is any proof for that or perhaps any disproof either. I don't know what physical chemists think about this idea, but the stickiness can be produced by very low doses compared with what is necessary to depolymerize DNA in most experiments in the test-tube. There has been no clear histochemical evidence that there is a change in polymerization of DNA in cells.

Swanson: However, there is a pronounced oxygen effect here.

de Hevesy: You and Dr. Pelc have stated that 35 r has an effect.

Howard: Yes, we got a reduction in the number of cells synthesizing DNA in a 12-hour period, certainly after a dose of 50 r. One can see a maximal effect there. But we know that this dose has a big effect also on the division, and while we have not done the appropriate timing experiment after 35 or 50 r, it seems quite possible that delays in division would explain that effect also.

Latarjet: I should like to add in answer to Swanson's question that Dr. Ephrussi-Taylor and I have some data on a purified DNA, according to which its inactivation by X-rays acting mainly through direct effect is not influenced by the presence of oxygen. In these experiments, protection against indirect effect was secured by 10 per cent yeast extract. The protection within the cell cytoplasm is certainly higher. Therefore, if we consider those lesions, such as chromosome breaks, which are oxygen-sensitive, we may say either (a) that they do not result from a primary effect of the radiation on DNA; or (b) that oxygen does not act at the level of a primary radiochemical change on DNA.

Swanson: This would be a metabolic event of some sort, and fits in with what we believe.

Howard: It is certain that in some cells, at any rate, division of the nucleus does not always determine division of the cell. These two things are separable in many cells, and perhaps the synthesis of DNA is also separable from the division of the nucleus.

Lajtha: I think that DNA synthesis and division are clearly separable. One can inhibit mitosis with colchicine and certain concentrations of heparin, and neither of them will inhibit DNA synthesis. The result will be polyploid cells and arrest of metaphase.

Spiegelman: I would like to suggest that one of the most useful systems that might be employed to study this phenomenon is a phased thymine-less mutant where you can control DNA synthesis, nuclear division, and examine for sensitivity. Here, many of the important parameters would be more or less under fairly precise control. In point of fact you can quite easily phase a thymine-less nucleus by controlling the thymine.

Alper: Dr. Howard, would you be prepared to apply the same reasoning to u.v. effects?

Howard: I understand that DNA synthesis can be immediately and finally stopped by u.v. irradiation, and in this respect it seems to act rather differently from ionizing radiation. Perhaps the reason is that the nucleic acid itself has such a high absorption.

Alper: It is known from Stapleton's work that the sensitivity of bacteria is quite different if you irradiate them in the stationary phase before they have started synthesizing anything at all, and just at the end of that when they are about to go into the log-phase. It certainly is tempting to feel that somehow this lack of sensitivity is due to the fact that those about to enter the log-phase have already got their DNA synthesized.

Hollaender: However, the story is different with u.v., where you have the opposite effect to that obtained with X-rays. You have a very high sensitivity immediately before they go into the log-phase. This may be a question of absorption which has never been determined.

Alper: I have found that if you take bacteria in the stationary phase and irradiate them, you get prolongation of the lag-phase, but if you irradiate the bacteria which are just about to go into log-phase and plot the growth curve after that, you get an increased lag-phase, and then you get the catching-up effect which I mentioned and very much less cell death.

Gale: May I ask if those cells are really not growing or are they just producing morphologically odd forms?

Alper: The experiments I have just mentioned were all done on viable counts. But I have also been doing some morphological work and get quite distinct dose-dependence curves, whether I am looking at the added lag, at the number of long forms produced, or the number of long forms that will go on and produce colonies.

Brachet: Dr. Howard, are you completely satisfied that ^{32}P, or any other precursor you are using in the autographic method, is really an indicator of the time of DNA synthesis? Can you rule out any turnover of DNA? There is also the problem of the constancy of the DNA content of the nucleus; I am quite willing to think that it is approximately constant. I am willing, also, to think that DNA is relatively stable, but I am not absolutely convinced that DNA is completely inert, and that it is always entirely constant.

Howard: In answer to your first question, I think that from autoradiographic work which Dr. Pelc and I did with the bean root, the period of uptake of ^{32}P into DNA is reasonably in agreement with the period during which the DNA is increased. But as regards the adenine labelling in the ascites tumour the situation is much less clear, and there is a good possibility there that the time at which the DNA is labelled with adenine does not coincide with what one can observe biochemically, i.e. an increased amount of DNA in the cell. This is still an open question, because the biochemical results seem to be in serious conflict with each other.

With regard to the constancy of DNA per chromosome set (I think we should say that, rather than per nucleus), there seem to be a few exceptional cases in which too much or too little DNA is found, to be consistent with this theory. But the exceptions are rather few, and I feel fairly satisfied that it is a general rule that the chromosome carries an amount of chromosomal DNA which is fixed for that chromosome. It would take a good deal more evidence than now exists to overthrow

that idea. Some cells may be producing DNA as a sort of secretion product, and in that case one might find more DNA or perhaps DNA of a different kind, or a different degree of polymerization, in such cells; but these would also be exceptional cases.

Brachet: It appears that some workers do not quite agree with this view, which is held by most American workers. Dr. Fautrez came to the conclusion that, with the same apparatus, he can get different results under different physiological conditions.

Howard: I know of Fautrez' work, and I agree entirely that one should not be dogmatic, but still I think it is up to him to prove his point, because at least in some of his work insufficient allowance has been made for synthesis due to preparation for mitosis. One has to have a pretty complete knowledge of the changes in cell population that are going on over a period of time to exclude this reason for different DNA values, and this has not been sufficiently allowed for.

Davidson: I think it is true that in those cases where there are deviations from what one might call the Boivin-Vendrely rule, if I may use the term, the cells have been put under quite abnormal conditions, and that if you stick to physiological conditions the rule does follow fairly well. It is impossible to generalize completely, but on the whole I think the amount of DNA per chromosome set remains unchanged under ordinary physiological conditions.

There is one point I would like to make in relation to Prof. Brachet's earlier remark about ^{32}P incorporation into DNA. We have recently been doing a lot of work on incorporation of various precursors into ascites cells *in vitro*, and the situation there is that under the conditions employed there is excellent incorporation of ^{32}P into DNA, good incorporation of [8–^{14}C]adenine, but next to no incorporation of labelled formate or labelled glycine; this suggests that purine synthesis just does not occur, and presumably DNA synthesis does not occur to any appreciable extent either, although it is very difficult to measure the total amount of DNA because the increase one would expect would be so small as to be within the experimental error of the estimation. There is no doubt whatever that ^{32}P is in fact incorporated into the DNA, because we have degraded the DNA to the individual deoxynucleotides and separated them and found incorporation into each individual nucleotide, just as into the whole DNA.

Spiegelman: Some very careful experiments have been done recently by Siminovitch, using cultures of bacteria as well as tissue, in an attempt to detect such a turnover, and he finds none.

Gray: Prof. Davidson, I infer from what you say that you thought that there was no cell growth going on in your *in vitro* preparations. Is this the point of your remark?

Davidson: It would appear that there is no *de novo* purine synthesis going on, as indicated by lack of incorporation of formate and lack of incorporation of glycine. There is good incorporation of formate into the thymine of the DNA, as Totter found with marrow cells *in vitro* (Totter, J. R. (1955), *J. Amer. chem. Soc.*, **76**, 2196). Incorporation into the methyl group of thymine is excellent.

Holmes: Could it be that you have got enough adenine present and that the cells are simply using that; some cells use it preferentially, and don't synthesize *de novo*?

Davidson: We consider this unlikely since the pool of acid-soluble adenine compounds in the ascites cells seems to be small. Indeed we are inclined to regard these cells as parasites on the purine-synthesizing mechanisms of their hosts. They can, of course, utilize intact purines; that does happen.

Lajtha: It must happen, because we gave some aminopterin in low concentrations to cultures, and this prevents the ^{14}C-formate incorporation into thymine, but it did not prevent the ^{14}C-adenine incorporation into DNA. Now since we were not prepared to believe that these cells synthesize a thymine-less DNA, we thought that there must be a pool of thymine and that there must be a pool of adenine as well, so that if we prevent the incorporation of labelled formate the cells can still use their preformed pool substances.

Davidson: There is one interesting point here, and that is that in the ascites cells which normally do not incorporate formate *in vitro* to any appreciable extent into the DNA purines, the addition of a particle-free saline extract of liver cytoplasm will very markedly stimulate the incorporation of formate into the purines of both RNA and DNA.

Howard: We all agree that the usefulness of the autoradiographic method depends on biochemical analysis and biochemical identification of the compounds, and this is not a very easy matter on which organic chemists can at once agree. On the other hand, the autoradiographic method is the only tracer method of looking at individual cells and this seems to be a very important thing to do. Therefore, the autoradiographer is in the biochemist's hands for advice on the identity of the compound.

Alexander: Is there sufficient data to make a clear distinction between interphase cell death and cell death following division? Can the possibility be completely excluded that cell death occurs on average at a time after irradiation which depends on the size of the dose?

Howard: We can be fairly certain that there are these two kinds of cell death.

Lajtha: With regard to Prof. Brachet's remark about the possibility of exchange, we calculated the number of molecules getting into the cell, into DNA, and both with ^{14}C-adenine and with ^{14}C-formate we got identical numbers as far as the technique allows: of the order of 20 million per cell DNA. Since this is valid both for thymine and for adenine, if that had been exchange that would imply exchange of 20 million adenine-thymine pairs, and I find it very difficult to believe that such an extent of exchange can happen if we accept the helical structure of DNA.

THE INFLUENCE OF RADIATION ON THE METABOLISM OF ASCITES TUMOUR CELLS*

ARNE FORSSBERG
Institute of Radiophysics, Stockholm

FOR some years we have been concerned with studies on effects of X-rays on Ehrlich ascites tumours. Some of that work is included in the present survey.

When an intraperitoneally growing Ehrlich ascites tumour in a stage of rapid growth is irradiated *in vivo* with a dose of 1250 r, comparatively moderate cell lesions arise (Klein and Forssberg, 1954). From analyses during a 48-hour observation period following that dose it appears that the number of tumour cells is not significantly increased nor does the percentage of non-tumourous exudate cells increase. The first property is due to the fact that cell division is completely inhibited and that the slow reappearance of mitotic activity which occurs some 20 hours after irradiation does not appreciably increase the total number of tumour cells in the sample. The second property is at variance with the findings when doses of the order of LD_{50} or thereabouts are administered. A relative increase in exudate cells is *inter alia* provoked by the presence of disintegrating tumour cells. The constant cellular composition of the sample is a prerequisite for the present series of investigations because it enables biochemical studies to be made on a cell population at various times in the interval 0–48 hours after irradiation, under well defined conditions. Furthermore, judging from the method of supravital staining as well as from biochemical properties, irradiation does not significantly increase the number of dead cells. Although the cellular composition is fairly constant, cytological changes do occur. Mitosis disappears during the

* Review based on work in co-operation with Drs. G. Klein and L. Révész, Institute of Cell Research, Karolinska Institutet, Stockholm.

first two hours following irradiation, and renewed cell division which appears at 20 hours must be preceded by a period of preparation well before that time.

Determinations of DNA, calculated per single cell, indicated a slight increase only during the 48-hour postirradiation period, whereas total N and RNA increased considerably. Almost as a consequence of the rather unimpaired synthesis of the cellular constituents (with the exception of DNA), the average cell volume also showed a progressive increase. It is interesting to compare the rate of cellular enlargement and synthesis of N and RNA of the irradiated cells with the rate of cell multiplication in the non-irradiated tumour. It appears that all these measures are the same within the limits of error of determination. In other words, this implies that the production of cell mass in an irradiated sample occurs through an increase in the mass of single cells while in the non-irradiated population the cell mass production during the same period is due to division, producing cells of ordinary size.

A survey of the current literature reveals a number of difficulties with regard to the quantitative interpretation of radiation effects when doses of the order of LD_{50} are given. An example of this is provided by growth rate studies on Ehrlich ascites cells irradiated *in vitro*: when mice were inoculated with the irradiated cells and the cell multiplication assayed (Révész, 1955), results were obtained which suggested that decay products from X-ray-killed and lysed cells may serve the survivors as an additional substrate, thus enhancing the growth rate. Furthermore, growth rate studies of artificial mixtures of X-ray-killed and living cells gave similar results. To arrive at more precise information on the effects of lethal doses in the present case, model experiments were designed and preliminary results may be mentioned here. The stimulation to enhanced growth at certain dose levels could be due to a general increase in the pool of metabolites, arising from the disintegration of dead cells; alternatively some particular products may be more effective in this respect. For several reasons, nucleic acids are the most interesting in this connection.

When DNA suspensions, isolated from Ehrlich ascites and purified from proteins, were injected intraperitoneally in quantities of about 0·5 mg. in Ehrlich ascites of mice which had been irradiated in the manner described, mitotic activity reappeared in the tumour cell population somewhat earlier and the number of cells in mitosis was higher than in samples from animals treated by irradiation and saline injection only; whether the DNA injection was given 2 hours before or 2 hours after irradiation, the effect was approximately the same. In similar experiments with DNA isolated from calf thymus or mouse liver no stimulating effect has so far been observed.

Since the ascites fluid contains DNAse, injected DNA can be expected to be enzymatically degraded at a rapid rate, and a species specific character of the DNA should be lost. Gale (1955) has shown that incorporation of amino acids in *Staphylococcus aureus* is inhibited in cells disrupted with ultrasonic treatment and deprived of their nucleic acids, but that this faculty can be restored by adding either homologous DNA or RNA. If the nucleic acids were enzymatically degraded, reactivation of amino acid incorporation took place even after addition of heterologous nucleic acid degradation products, e.g. from yeast. In view of these results, the mechanism of the DNA effect in our experiments is so far obscure.

One would expect the stimulation caused by Ehrlich DNA to be rather transient as the depôt is probably used up within a short time, in contrast to what happens when X-ray-killed cells are mixed with living cells. It can be assumed that the lysis of dead cells is protracted over a fairly long period and the material furnished—not only DNA but also other cellular constituents—is available during correspondingly longer intervals of time.

It can be readily demonstrated by isotope-labelling methods that DNA from X-ray-killed cells, or at least important parts of the DNA molecule, can be transferred to living cells of the same sample during growth. We used cells which were labelled *in vivo* with ^{14}C-adenine, and harvested the cells some days after injection when 97 per cent or more of the adenine had

been incorporated into DNA and RNA. ^{14}C-labelled cells were X-ray-killed and mixed in various proportions with unlabelled living cells, and mice were inoculated with these mixtures. Analyses made on the fifth day after inoculation, when control experiments showed that all X-ray-killed cells were completely lysed, showed consistently high incorporation of activity into the living cells, e.g. when a mixture with a ratio of living: dead cells = 1: 1 was used, about 40 per cent of the activity was incorporated into the living cells, and the activity was found to be distributed between DNA and RNA in the same proportions as in the inoculated sample.

This transfer of activity and, thus, of metabolites from dead to living cells is not unexpected but, nevertheless, it does not seem to have received much consideration in radiobiological work. The extent to which, and in particular, how soon after administration of the dose such a transfer takes place, is still uncertain. It would seem, however, that in studies with labelled compounds, carried out several days after administration of an LD_{50} dose, such secondary reactions cannot be ruled out.

In recent years, considerable work has been devoted to the question of whether compounds like DNA, RNA and proteins are metabolically stable and do not undergo concentration changes during cell division and growth. Several observations indicate that DNA from unicellular organisms is equally distributed between the daughter cells by mitosis without previous degradation and that no replacement occurs in the resting cells; this seems to be the case e.g. in bacteria (Hershey, 1954). In higher organisms the stability of DNA is more controversial (Hevesy, 1948; Stevens, Daoust and Leblond, 1953; Barnum, Huseby and Vermund, 1953), but the evidence of some investigators favours this concept (Barton, 1954; Fujisawa and Sibatani, 1954). The stability of RNA as well as of DNA was advocated by Nygaard (Nygaard and Rusch, 1955) from experiments on regenerating liver. The proteins of β-galactosidase from *Esch. coli* were found by Hogness, Cohn and Monod (1955) to be static. Evidence to the contrary has been put forward even in the

case of RNA and proteins. Since the total activity of Landschütz ascites cells, labelled with [2-^{14}C]glycine, is diluted by cell multiplication more than could be expected from increase in cell mass, Greenlees and LePage (1955), for instance, deduced an exchange (loss) of protein-bound activity amounting to about 9 per cent per day. Using the same material,

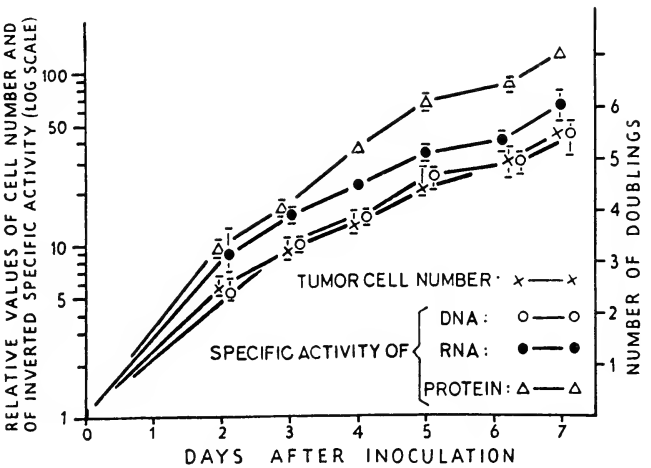


FIG. 1. Total number of free tumour cells and inverted specific activity of DNA, RNA and proteins as a function of time after inoculation. All values were brought to a common scale (left) by taking the values corresponding to the inoculum as equal to one. Curves are drawn through the geometrical mean of the values from the different series, each value representing a pool of ascites from 4 to 15 mice. The right-hand scale shows the number of generations.

Ledoux and Revell (1955) made the observation that the RNA concentration per cell decreased considerably with the age of the inoculated cell culture.

Ehrlich tumour cells, with [8-^{14}C]adenine or [2-^{14}C]glycine as precursors, were used in our studies on similar stability problems (Révész, Forssberg and Klein, unpublished). In parallel analyses of specific activity and cell multiplication during the week following inoculation of the cells labelled *in vivo*, we obtained the results shown in Fig. 1. There is a

close agreement between the growth curve and the dilution of DNA activity. The corresponding data for RNA and proteins show appreciable deviations from expectation assuming stability. However, an important difference is that in the case of proteins a progressive loss of activity through

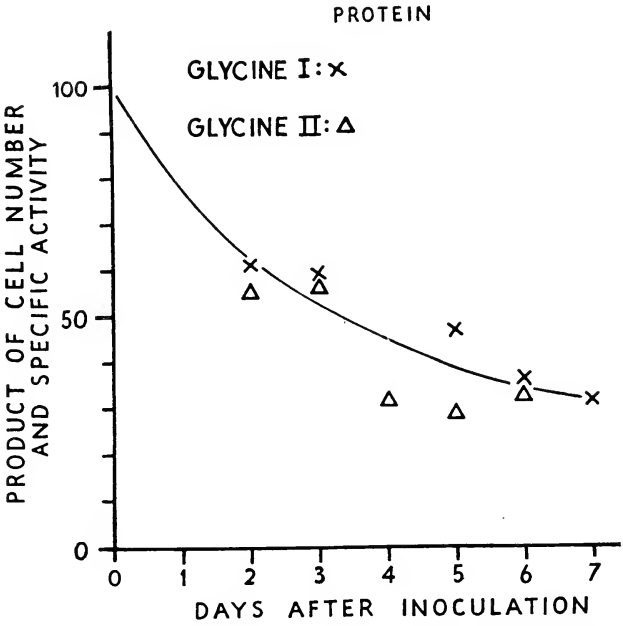


FIG. 2. Products of total number of free tumour cells and specific activity of the protein fraction. The initial value is taken as equal to 100. Two series of experiments.

exchange and release from the once incorporated ^{14}C-glycine occurs (Fig. 2), as compared to an initial deviation only in the RNA curve taking place between the collection of labelled cells and the second day of growth of the inoculate. The continuous loss of protein-bound ^{14}C is in agreement with the observation of Greenlees. The activity released from cell proteins is, however, not in a chemical form which can be used for further synthesis of nucleic acids. We are led to this

conclusion by the fact that the specific activity curves of RNA and DNA agree in the series labelled with ^{14}C-glycine and ^{14}C-adenine. In the latter case no marking of the proteins or the tissues of the host takes place.

The ratio of the specific activities of RNA: DNA is comparatively constant during the whole period of observation of the transferred inoculates, and the mean value of 0·72 is seemingly independent of whether adenine or glycine is used as precursor (Table I).

Table I

RATIOS OF THE SPECIFIC ACTIVITIES OF RNA: DNA IN THE INOCULUM AND AT VARIOUS TIMES AFTER INOCULATION

Inoculum	*Days after inoculation*	*Glycine I*	*Glycine II*	*Adenine I*	*Adenine II*
Glycine I: 1·11	2	0·60	0·69	0·56	0·72
Glycine II: 0·97	3	0·81	0·69	0·58	0·69
Adenine I: 0·95	4	—	—	0·64	0·93
Adenine II: 1·01	5	0·79	0·80	0·59	0·78
	6	0·87	0·91	0·52	0·81
Mean 1·01	7	0·73	—	0·60	0·75
	Mean:	0·76	0·77	0·58	0·78
	Mean and standard error of all four experiments: 0·72 = 0·025				

Furthermore, in the inoculum sample at zero day the specific activity ratio is 1, and earlier still, i.e. shortly after injection of the labelled compound, values ranging from 1·5–4 are found.

This decrease in the RNA: DNA specific activity ratio and the eventual attainment of a steady state calls to mind the similar results of Ledoux and Revell which, however, were arrived at from determinations of the total amounts per cell in the related Landschütz tumour. Assuming that similar conditions prevail in the Ehrlich cell, the initial loss in RNA

activity (Fig. 1) might be due to a loss of highly active RNA molecules.

During the first two hours after irradiation, the incorporation of [2-^{14}C]glycine into both DNA and RNA was depressed to the same degree, averaging about 70 per cent of that of the non-irradiated tumour cells in our experiments. This was established through analyses in which glycine was injected immediately after radiation and the tumour cells were assayed at various times in the interval 0–120 minutes (Forssberg and Klein, 1954). Intraperitoneally injected glycine is very rapidly taken up by the cells. Five minutes after injection, the uptake was found to be the same in irradiated and control cells; the decreased incorporation of glycine in the nucleic acids is therefore not caused by any changes in permeability.

On comparison of the purely chemical analysis of RNA and DNA with isotope determinations, it appears that total RNA synthesis proceeds roughly linearly with the production of cell mass during the 48-hour period, whereas isotope incorporation, as stated, is reduced to 70 per cent of the normal value during the first two hours. DNA incorporation of ^{14}C is also reduced to the 70 per cent level as contrasted to the increase in the total amount of DNA which proceeds at an average of 33 per cent of that of the unirradiated controls.

In the amitotic period which follows (time interval 2–20 hours), marked changes in the incorporation rate of both RNA and DNA take place and specific activity values equal to or even higher than those of the controls occur. These results were obtained in experiments where ^{14}C-glycine was injected over a period of two hours at different times during the amitotic period. As far as incorporation rate is equivalent to synthesis, this ought to imply that the initial depression of synthesis is followed by a period of increased synthesis.

In the case of RNA, the increased incorporation rate subsequent to the initial depression shifts the balance, so that the isotope measurement can be made compatible with the finding that the total RNA is synthesized for the most

part at a normal rate. The fact that isotope incorporation into DNA during the same period is somewhat increased as compared with the initial rate, and occasionally also indicates activities higher than those of the controls, still more emphasizes the discrepancy between total determinations and isotope measurements. Similar indications of an overcompensation in the incorporation of isotopes into DNA have

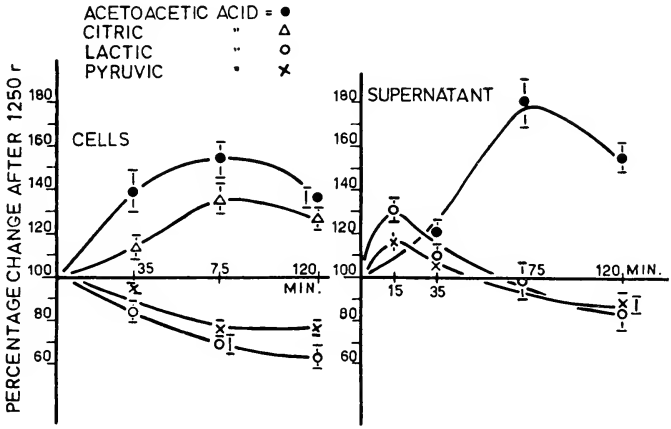


FIG. 3. Changes in the cellular concentration of acetoacetic, citric, lactic and pyruvic acid in Ehrlich ascites cells. Dose 1250 r. The concentration in non-irradiated cells is taken as equal to 100 (left). Corresponding changes in the ascites fluid ("supernatant", right). Determinations during the first two hours after irradiation.

been reported also when lethal doses were administered (Kelly *et al.*, 1955).

Protein synthesis in our material is never appreciably influenced by irradiation, although isotope measurements indicate a slight depression of synthesis during the first hours after irradiation, but a slight increase at approximately the same time as the measurements indicates that DNA and RNA activity is high. A period of general recovery is indicated by all these findings. Simultaneously also, the metabolic changes discussed below (Fig. 3) seem to level out.

When the mitotic activity is brought to a stop, a number of

cellular reactions other than those already mentioned are proceeding at an irregular rate; e.g. studies in progress of the intermediate carbohydrate metabolism indicate reversible changes in the concentration of various acids (Fig. 3). It appears as if the citric acid consumption is blocked for a short period of time, leading to increased cellular concentration. If so, the blocking is a temporary one, as indicated by the slope of the citric acid curve as well as by occasional analyses which showed almost normal citric acid values some hours later. The changes in acetoacetic acid concentration during these first two hours after irradiation proceed in a similar fashion.

Simultaneously, the concentration of pyruvic and lactic acid decreases. This may be due in part to a blocking of carbohydrate metabolism. Determinations in the ascites fluid (Fig. 3, "supernatant") demonstrate that the latter acids are also partly released from the cells into the peritoneal fluid and thence from the entire ascites. Similarly, also, the fluid from irradiated samples is richer in acetoacetic and citric acid (values for the latter are not included in the curve). This leakage renders quantitative determination in the cells rather difficult. Two to four hours after administration of the dose, ^{14}C-labelled lactic acid was found to be incorporated into liver glycogen; thus, at least part of the lactic acid is stored in the irradiated liver. Increased incorporation of ^{14}C from labelled glucose into the liver glycogen from ordinary irradiated mice has been reported by Lourau (1955).

As a result of *in vivo* irradiation the host animals also receive the same dose as the ascites cells. It is of interest to note that neither the intestines nor the liver show any significant deviation from their normal state with respect to the concentration of acetoacetic, lactic and pyruvic acid during this period of observation. Citric acid determinations were highly variable but, on an average, were slightly increased in the irradiated liver. Changes in the citric acid metabolism have been reported by DuBois, Cochran and Douall (1951), but on animals which had been both fluoroacetate poisoned and irradiated.

REFERENCES

BARNUM, C. P., HUSEBY, R. A., and VERMUND, H. (1953). *Cancer Res.*, **13**, 880.
BARTON, A. D. (1954). *Fed. Proc.*, **13**, 422.
DUBOIS, K. P., COCHRAN, K. W., and DOUALL, J. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 422.
FORSSBERG, A., and KLEIN, G. (1954). *Exp. Cell Res.*, **7**, 480.
FUJISAWA, Y., and SIBATANI, A. (1954). *Experientia*, **10**, 178.
GALE, E. F. (1955). III Int. Congr. Biochem. p. 71, Liège: Vaillant-Carmanne.
GREENLEES, J., and LEPAGE, G. A. (1955). *Cancer Res.*, **15**, 256.
HERSHEY, A. D. (1954). *J. gen. Physiol.*, **38**, 145.
HEVESY, G. (1948). Advances in Biological and Medical Physics, **1**, 409, New York: Academic Press.
HOGNESS, D. S., COHN, M., and MONOD, J. (1955). *Biochim. biophys. acta*, **16**, 99.
KELLY, L. S., HIRSCH, J. D., BEACH, G., and PAYNE, A. H. (1955). *Radiation Res.*, **2**, 490.
KLEIN, G., and FORSSBERG, A. (1954). *Exp. Cell Res.*, **6**, 211.
LEDOUX, L., and REVELL, S. H. (1955). *Biochim. biophys. acta*, **18**, 416.
LOURAU, M. (1955). Radiobiology Symposium, p. 225, London: Butterworth.
NYGAARD, O., and RUSCH, H. P. (1955). *Cancer Res.*, **15**, 240.
RÉVÉSZ, L. (1955). *J. nat. Cancer Inst.*, **15**, 1691.
STEVENS, C. E., DAOUST, R., and LEBLOND, C. P. (1953). *J. biol. Chem.*, **202**, 177.

DISCUSSION

Popjak: I would like to make some comments regarding the rate of metabolism of ascites tumour cells, and the deductions one might make when measuring metabolic events *in vivo* compared to *in vitro*. Prof. Davidson mentioned that *in vitro* he gets excellent labelling with ^{32}P or with adenine, but no evidence for purine synthesis. *In vivo*, on the other hand, you get labelling from glycine and from formate. I feel that the *in vivo* labelling from glycine and from formate may not necessarily mean that purine has been synthesized within the ascites cell. We thought the ascites cell was a very convenient preparation for measuring certain problems of fat metabolism. We carried out some *in vitro* incubations, and found only minute traces of synthetic ability of these ascites tumour cells, e.g. *in vitro* they cannot synthesize fatty acids from acetate under conditions of, say, liver slices or mammary gland slices; that relates to your figures for acetoacetic acid, Dr. Forssberg. They can hardly oxidize acetate to CO_2, from which I must assume that the citric acid cycle is working at a very poor rate indeed. I wonder whether some of the *in vivo* incorporation data that one observes with the ascites tumour might not be due to the fact that the compounds are synthesized elsewhere and then transferred, because in the case of lipids,

for example, we observed that when we had labelled lipids in the form of egg-yolk then it all appeared very nicely in the ascites tumour.

Forssberg: May I ask if you are doing these experiments in an atmosphere of oxygen, because our experiments were carried out under rather anaerobic conditions.

Popjak: These were aerobic, not in pure oxygen but in air.

Forssberg: According to Christensen and Riggs (1952, *J. biol. Chem.*, **194**, 57) there is a rapid uptake of amino acids in ascites cells against a strong concentration gradient. We ourselves found that 5 minutes after an intraperitoneal injection the uptake was already very high and was quantitatively similar in X-rayed and control cells. The glycine seems to enter the cells directly from the intraperitoneal injection.

Popjak: I am not suggesting that this might be the case for all cell constituents, but it might be that in certain cases the preformed substances from plasma are taken out through the ascitic fluid.

Krebs: Your last slide rather suggests that the conversion of lactic and pyruvic acid into acetic acid is increased, and the two products of acetyl coenzyme A, acetoacetate and citrate, are present in higher concentration. I wonder whether any other experiments would be in agreement with this conclusion, that you get a more rapid oxidation.

Forssberg: Oxygen tension in the ascites fluid is rather low. On a molar basis the concentration of acetoacetic acid seems to be higher than that which corresponds to a condensation of pyruvic acid. It appears, however, from the analysis of the fluid that these substances begin to leak from the cells almost immediately after irradiation; therefore, quantitative determinations are uncertain. Mouse liver and intestines which are irradiated at the same time as the ascites cells do not show these changes, or at least show them only to a minor degree. Whether any other similar experiments have been done, I do not know.

Krebs: The decrease is not in arithmetical proportion to the increase.

Lajtha: I think that the ascites cell cannot synthesize many things *de novo*. On the other hand, the transformation and the transport can be extremely quick. We gave ^{14}C-formate *in vivo* to mice, and within 45 minutes the ascites cells became so heavily labelled that they were useless for autoradiography. Not only DNA but also RNA and proteins were labelled. However, the same ascites cells in the same ascites fluid *in vitro* in 3- to 6-hour cultures did not show any uptake of ^{14}C-formate, except small amounts in DNA thymine. With regard to the very curious discrepancy between the depression in DNA synthesis and the labelling of the DNA, I wonder whether that could be explained by cells dying only in mitosis, and therefore no increase of DNA would be observed in total of mass, but nevertheless the same cells were not being inhibited in the synthetic period which is fairly long in these cells.

Forssberg: The cells are carrying out some vital functions, e.g. production of total cell mass (i.e. proteins and RNA), at a fairly normal rate during the first 48 hours after irradiation and are "living" as judged from vital staining; so I do not think there can be much cell death in mitosis during this period.

Lajtha: Is the cycle time constant in these cells?

Forssberg: Cycle time may be taken as approximately constant for a 48-hour observation period in controls. In irradiated samples mitosis disappears for about 15–20 hours. What the cycle time may be when mitosis appears, I cannot say.

Howard: Dr. Forssberg, since you have done experiments showing that labelled nucleic acid of dead cells can appear in the nucleic acids of growing cells in the tumour, and apparently can also stimulate division, do you regard this as something which might happen in a tumour which is given heavy doses of irradiation in which there must be a great deal of cell death, and do you think this would be a factor in the treatment of tumours with radiation? These dead cells are doing something to the metabolism of the living ones, in the way of stimulating growth; what significance has this in therapeutic treatment of tumours?

Forssberg: I think that in therapeutic treatment doses should be kept as high as possible in order to kill as many tumour cells as possible. In ascites cells these effects appear with fairly low doses. It seems to me that the increased labelling of DNA which Dr. Kelly found 2–3 days after irradiation could be caused by transfer from irradiation-killed cells.

Howard: This seems quite a possibility in many irradiated tissues.

Latarjet: Dr. Howard's question impels me to say a few words about the mysterious effect which Dr. Delaporte observed in 1949 after u.v. irradiation and which she has recently investigated after X-irradiation in my laboratory. She irradiated *Esch. coli* bacteria with about 80 kr., a dose which apparently leaves about 10^{-4} colony-forming cells when a heavy inoculum is plated on agar. If one looks at the plate under the microscope during incubation, one observes at the early origin of a colony not a single growing cell but a rather large number of enlarged growing cells undergoing a few divisions. Dr. Delaporte's idea is that once a surviving cell has started growing, surrounding "dead" cells, at a short distance of less than 100 microns, are induced into growth. Restorability would last a short time, thus limiting the process which otherwise would expand to the whole plate. This restoration would be very effective between clumped cells. As a matter of fact, after u.v. at least, once a cell starts growing within a clump, all the cells of this clump start growing too.

I must add that what is to me the fundamental question has not yet been cleared up, i.e. whether there is induction to cell enlargement followed by a few divisions (delayed death instead of immediate death), or to true restoration i.e. to indefinite cell multiplication. This question may be answered by distinguishing the restoring cell from the eventually restored ones. I am now using two mutants of *Esch. coli* for this purpose. The experiments have just begun. The only thing I can say at the moment is that, under my experimental conditions, using a high dose of 80 kr, the phenomenon of "neighbourhood restoration", if it does exist, is rare.

INFLUENCE OF RADIATION ON METABOLISM OF REGENERATING RAT LIVER

Barbara E. Holmes

Department of Radiotherapeutics, University of Cambridge

Any attempt to discover something of the biochemical reactions concerned in cell division or the effects of irradiation on the mitotic processes, is liable to lead to the necessity of dealing with single cells or cells dividing synchronously. The use of growing tissues is limited by the fact that all stages of the mitotic cycle are here present together and any estimation can only give an average value. Histochemical techniques have been evolved to make possible the examination of a single cell and the elegant autoradiographic methods of Pelc and Howard are among the most successful. For work on a larger scale the synchronously dividing tissue is useful and, among mammalian tissues, the regenerating liver is a convenient example.

Price and Laird (1953) and also Abercrombie and Harkness (1951), Stowell (1949) and others who introduced the experimental use of regenerating liver tissue, found that a large synthesis of DNA had taken place in the remaining lobes of a rat liver 24 hours after hepatectomy, whereas cell division had not yet begun. An opportunity of studying the chemical events leading up to mitosis was thus available. This tissue was used in our laboratory to examine the radiosensitivity of different stages of the mitotic cycle. Mrs. Kelly of the Donner Laboratory used carbon tetrachloride poisoning to cause partial destruction of liver cells, which was followed by regeneration, and estimated the effect of whole body irradiation on this. She found a very large increase in DNA synthesis in the liver, beginning at 30 hours, and reaching its peak at 36 hours after administration of the drug; a high rate of

mitosis was not seen until 12 hours later. The DNA synthesis could be inhibited by irradiation (800 r whole body) at 12 hours (not later than 24 hours) after poisoning, but even 2000 r could not inhibit the synthesis while it was actively in progress. Another sensitive period for this inhibition was

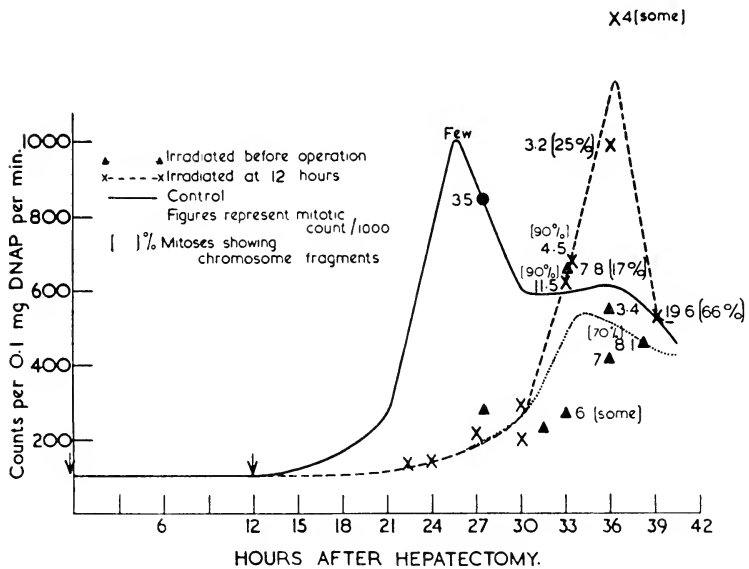


FIG. 1. Rate of DNA synthesis and mitosis in regenerating liver.

found by Mrs. Kelly to occur later than 60 hours after poisoning (Kelly *et al.*, 1955).

The curve of regeneration we obtained after hepatectomy (Fig. 1) has some different time relationships. The peak of the DNA synthesis rate seems to be shortly before the first large outburst of mitosis. As soon as large numbers of mitoses are present the rate is diminished.

A dose of 2000 r inhibits DNA synthesis by about 50 per cent at any time during the cycle and the effect is immediately apparent. Here our results differ from those obtained by

Mrs. Kelly. Small doses of irradiation, in our case 450 r, do not have any obvious immediate inhibitory action on DNA formation if given during synthesis, although they delay mitosis and cause chromosome breaks.

As it had been shown by the work of Pelc and Howard (1953) and of Kelly and co-workers (1955) that small doses given before the beginning of synthesis have a marked delaying action on DNA formation, we carried out irradiations with 450 r at 12 hours after hepatectomy. It can be seen from Fig. 1 that the increased rate of synthesis normally begins by 15 hours. The irradiation delayed the increased DNA formation and the onset of mitosis for about 10 hours; at the time when the control liver showed a very high rate of synthesis (at about 24 hours after hepatectomy) the difference between the control and the irradiated tissue was very great. We found that we could demonstrate the effect of 450 r of X-ray irradiation at any stage of liver regeneration if we waited 9–12 hours for the difference between the control and irradiated liver to become obvious. This gave direct confirmation of the work carried out by Pelc and Howard on the bean root with radioautographic techniques.

Recovery (or partial recovery), as measured by DNA synthesis and mitosis, comes at about 36 hours after hepatectomy. It is obviously difficult to say whether the cells concerned in synthesis and division are the same cells which were forced to delay their activity, or whether another group of cells has taken their place. It has been supposed that cells not yet preparing for DNA synthesis might be unaffected by the irradiation and that these undamaged cells could enter into DNA synthesis and mitosis in their turn, causing the apparent recovery. We have been able to show that this is not the case. Irradiation with 450 r *before* hepatectomy shows the same inhibition and recovery at the same time (Fig. 1) as are shown by irradiation 12 hours after, so that it is not possible to picture an early interphase stage which is unaffected by the irradiation. In three cases irradiation was carried out 24 hours before hepatectomy; here there is some recovery in the

sense that mitoses occur at 27–28 hours after the hepatectomy. These are fewer than in the control and show many chromosome breaks and the DNA synthesis rate is lower. It is plain that this question of the irradiation of the resting liver tissue should be further investigated.

It is worth mentioning that Dr. Koller, who carried out cytological investigations on squashes of the irradiated liver material, found a very high chromosome breakage rate (percentage numbers in Fig. 1) in the belated mitoses after irradiation at 12 hours after hepatectomy. This early stage is not usually supposed to be a sensitive stage as regards the production of chromosome breaks. (All the mitotic counts were done by Dr. D. Cater.)

In much of the work on regenerating liver, described in the literature, whole body irradiation is used. For our experiments, irradiation was given immediately over the area of the right hand liver lobe and the rest of the abdomen was screened with lead rubber. In early experiments we removed food from the cages and gave both control and irradiated animals injections of glucose saline. More recently we have found that the irradiated animals will eat quite well and we have not taken any such special steps. Animals showing any adverse symptoms, or lack of muscular tone, or which have lost an unusual amount of blood at operation or have subsequently injured the muscle scar by struggling, cannot be used. These conditions cause delay in regeneration.

In experiments with regenerating rat liver, as with Jensen rat sarcoma, it is found that irradiation inhibits the ^{32}P uptake into DNA but not into RNA. Ord and Stocken (1956) agree with this, but point out that in some tissues RNA synthesis is also affected. Abrams (1951) found some inhibition of ^{32}P uptake into the RNA fraction of some mouse tissues after whole body irradiation. In normal as well as regenerating liver Kelly (1952) has found that whole body irradiation will cause a depression of ^{32}P uptake into nuclear RNA but an increase in cytoplasmic RNA.

Kelly and Payne (1953) also studied the effect of whole

body irradiation on the incorporation of adenine in the nucleic acids of various tissues. About 60 per cent depression of incorporation into DNA was found 1–3 hours after irradiation while the effect on RNA was slight. In most tissues, the depression of DNA synthesis after 48 hours was very large, whereas in the intestine the synthesis is actually much above normal at this time.

On the whole, the DNA synthesis is most affected in all tissues, which made it reasonable to consider the possibility that the synthesis of thymine or the thymine nucleotides of DNA might be particularly sensitive to irradiation. An attempt to show this was made by Mee (1956), who used [^{14}C] formate to follow the synthesis of the bases in RNA and DNA and obtained a regeneration curve which followed the ^{32}P uptake curve almost exactly. The bases were separated and the activity was estimated. Three hours after a dose of 2000 r, the specific activity of the DNA was depressed to half of the control value; the adenine, guanine and thymine were, however, equally affected. The possibility still remains, of course, that lack of thymine has prevented the appearance of half the control amount of new DNA. Ord and Stocken (1956), however, comparing the separated nucleotides of DNA in tissues irradiated *in vitro*, have given data which suggest that the addition of phosphorus to the purine nucleotides is more easily inhibited than the uptake of pyrimidine nucleotide phosphorus.

So far, this work had been concerned with changes in the nucleic acid metabolism only, but it was plainly of interest to relate changes in the general metabolism of the liver to these special ones. An investigation of the changes in metabolism accompanying regeneration and the effect of irradiation upon them has been carried out by Dr. Itzhaki of our laboratory and will be described in the following pages.

It has been known for some time (Ludwig, 1939; Gursch, Vars and Rardin, 1948) that the neutral fat content of regenerating liver was considerably above the normal level, but this rise in neutral fat had not been correlated with any

particular stage of regeneration. Table I shows that a large increase in fat content occurs soon after hepatectomy and persists for 2 days. It is not particularly connected with any stage of the mitotic cycle and may merely be a sign of altered or curtailed activity of the liver. The fat was extracted and estimated by the hydroxylamine and ferric chloride method of Stern and Shapiro (1953).

Table I

Fat Content of the Liver

Liver	*Time after hepatectomy*	*Number of animals*	*Fat (Per cent)*
Normal		10	3·64
Regenerating	15 hours	4	7·42
Regenerating	20 hours	3	5·86
Regenerating	26½ hours	10	7·30
Regenerating	2 days	5	5·96
Regenerating	5 days	4	4·01
Regenerating	9 days	1	3·50

The respiration of the liver was known to be higher after hepatectomy, and Schwartz and Barker (1954) had measured it at different times after the operation. Table II shows Itzhaki's data, which can be considered in connection with the synthesis and mitosis curves. The respiration is already very high at the beginning of synthesis, is unaltered during the period of high mitosis and continues to be high for 3 days. The respiration was measured in a Warburg apparatus in the presence of an excess of glucose.

The oxidation of glucose itself was estimated by measuring the specific activity of CO_2 derived from the oxidation of glucose uniformly labelled with ^{14}C. The CO_2 was trapped in potassium hydroxide, sodium carbonate was added as carrier and the carbonate precipitated as the barium salt, the weight of the barium salt being always about 100 mg. The factor for converting the counts, estimated to the total count, was known.

The total activity of the CO_2 is given as a percentage of the total activity of the glucose of the medium. The oxidation of glucose, as measured by this method, includes the direct oxidation of C_1 and the oxidation of the derived three-carbon molecules through the Krebs cycle.

Fig. 2 shows an increase in total glucose oxidation after hepatectomy. This can be seen here related to the other metabolic changes and to the mitotic cycle.

Table II

Oxygen Uptake by Liver Slices

Liver	*Time after hepatectomy*	*Number of animals*	*Oxygen uptake ($\mu l.O_2$/mg. fat-free dry tissue)*
Normal		10	6·67 ± 0·18
Regenerating	14 hours	3	9·15
Regenerating	20 hours	5	9·03 ± 0·81 *
Regenerating	26½ hours	8	9·00 ± 0·51 *
Regenerating	46 hours	3	9·51
Regenerating	3 days	1	9·40

The slices were incubated in Krebs-Ringer phosphate solution containing 0·4 per cent glucose.

* Statistical comparison shows that these figures are significantly higher than normal.

The comparison of samples of glucose specifically labelled in the 1- and in the 6- position shows that both the direct oxidation of glucose which results in the liberation of CO_2 from C_1 and the oxidation through the Krebs cycle, which results in liberation of CO_2 from C_6 as well as from C_1, are very definitely increased after hepatectomy. These figures will be published later by Dr. Itzhaki.

A large number of measurements of respiration and of fat content were made at 26 hours after hepatectomy and the mitotic rates were measured by Dr. Cater. No connection between the mitotic count and the respiration or fat content could be demonstrated.

Although it is well known that X-ray irradiation tends to have very little immediate effect on the general metabolism of a tissue, it did seem possible that an irradiation dose capable of delaying the regeneration of the liver might also

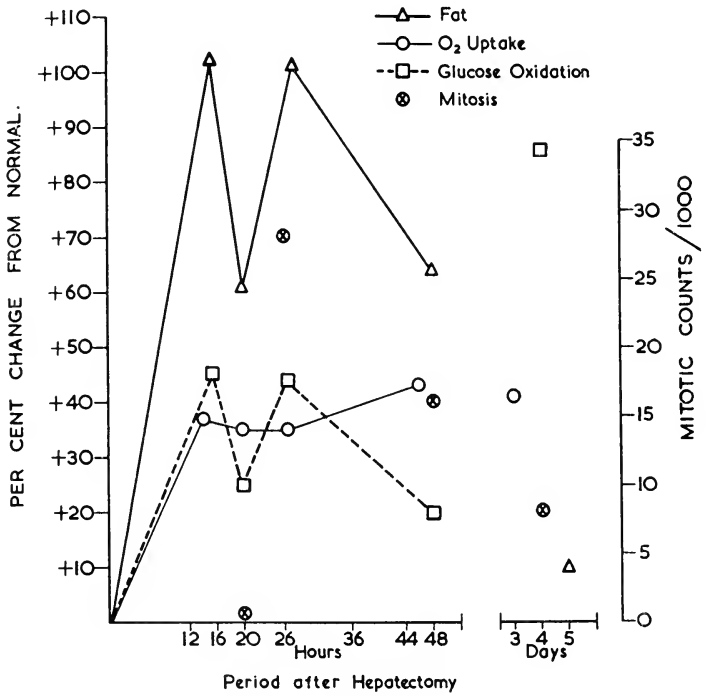


FIG. 2. Fat content, oxygen uptake and glucose oxidation of regenerating liver.

delay the appearance of increased respiration and increased fat. This, however, was not the case, as Table III shows, even when the rat was irradiated before hepatectomy.

The glucose oxidation, as measured by CO_2 output in the manner already described, gave somewhat different results, which are shown in Table IV. The oxidation of glucose by normal liver was unchanged by irradiation, but the increase in glucose oxidation found during early regeneration could be

inhibited by 450 r X-rays given before hepatectomy. The glucose oxidation has, in fact, been prevented from rising above the normal level in animals which are killed at 15 or 16 hours after hepatectomy, which is the only time interval so far used. The glucose oxidation at the time of recovery from irradiation must obviously be measured as soon as possible.

Table III

EFFECT OF RADIATION ON OXYGEN UPTAKE AND FAT CONTENT OF REGENERATING LIVER

Time after hepatectomy			*Oxygen uptake* ($\mu l.\ O_2/mg.$ *fat-free dry tissue*)	*Fat* (*Per cent*)	*Mitotic counts per* 1000
Irradiation		*Killing*			
450 r	Preoperation*	20 hours	8·43	5·42	
0	—	20 hours	8·20	6·00	
450 r	6 hours	23 hours	12·55	5·36	
450 r	12 hours	26 hours	10·00	7·33	0
0	—	26 hours	10·00	7·00	63
2200 r	23¼ hours	26¼ hours	11·20	6·03	0
0	—	26¼ hours	10·80	11·90	8
2200 r	20¼ hours	26¼ hours	9·56	7·95	0
0	—	26¼ hours	10·90	9·50	34

Values for normal liver: O_2 uptake 6·67, fat content 3·64.

* This rat was irradiated immediately before hepatectomy.

All these measurements were calculated on the basis of the weight of fat-free dry tissue, since the fat content was sometimes very high in the regenerating tissue. The effects of X-rays on glucose oxidation will be published in more detail later.

In the course of the irradiation experiments just described, it could always be shown that 450 r given during or at the end of DNA synthesis, although too late to prevent the synthesis, still had a marked delaying and damaging effect upon mitosis. We hoped to demonstrate, by another type of experiment, a

Table IV

EFFECT OF RADIATION ON GLUCOSE OXIDATION BY SLICES OF REGENERATING AND NORMAL LIVER

Experiment No.	*Yield of $^{14}CO_2$/100 mg. fat-free dry tissue (Per cent of total radioactivity of glucose)*			
	Regenerating liver		*Normal liver*	
	Non-irradiated	*Irradiated*	*Non-irradiated*	*Irradiated*
1	2·82	2·60	1·84	
2	2·14	1·46	1·29	
3	3·06	1·63	2·12	
4	2·44	1·77	1·83	2·07
5	2·27	1·82	1·69	1·40
6	2·14	1·85	2·06	1·49
7	2·16	2·33	1·84	1·93
8a			1·86	2·12
8b				2·08
9	3·42	2·27	1·99	
Mean ± S.E.	2·56 ± 0·17*	1·97 ± 0·14	1·84 ± 0·08	1·85 ± 0·13
No. of animals	8	8	9	6
Group	A	B	C	D

* Statistical comparison shows that the mean of group A is significantly higher than that of group B ($P = 0·02$) and that of C ($P < 0·01$).
Rats were killed 15½ to 16 hours after X-irradiation.
Irradiation dose was 600 r except in experiments 1 and 2 where the dose was 450 r. Partial hepatectomy was carried out immediately after irradiation.

more direct connection between inhibition of growth and inhibition of DNA synthesis by irradiation.

Dr. Dittrich, of Dr. Schubert's laboratory in Hamburg, was kind enough to send a strain of Ehrlich mouse carcinoma which had been made radioresistant by irradiation at a number of successive passages, as described by Dittrich, Höhne and Schubert (1956). This tumour was grown as a solid tumour in the leg of an inbred strain of white mice and was compared with a normally sensitive strain of the same tumour grown in the same strain of mice. Tumours inoculated on the same day and grown to the same size were used, and ^{32}P was

used as a tracer to estimate nucleic acid synthesis. Table V shows the results. At 2000 r, which was the dose used by Dittrich and Höhne to demonstrate the decrease in sensitivity,

Table V

X-RAY IRRADIATION OF EHRLICH MOUSE TUMOUR

Specific activity of DNA phosphorus as per cent of specific activity of inorganic phosphorus.

Dose	*Time after irradiation*	*Usual strain*		*Radioresistant strain*	
		DNA	*RNA*	*DNA*	*RNA*
2000 r	1½ hours	1·0	3·6	0·65	5·0
0	1½ hours	1·6	6·0	1·7	6·0
2000 r	1½ hours	0·5	3·2	0·6	3·0
0		1·65	5·0	1·25	4·5
2000 r	1½ hours	0·83	4·6	0·85	4·65
0		1·85	6·5	1·7	6·2
2000 r	1½ hours	1·9	6·5		
0		2·35	9·1		
2000 r	2 days	0·19	2·5	0·2	4·0
0		0·85	3·7	1·34	5·4
1650 r	1½ hours			1·5	10·0
0				1·75	6·5
1650 r	1½ hours	0·8	5·0	1·25	8·15
0		2·15	6·4	2·7	7·2
1500 r	2 days	0·25	4·1	0·33	4·8
0		0·9	4·35	0·75	3·65
1350 r	1½ hours	1·26	7·6	0·89	6·0
0		1·5	6·0	0·55	3·3

we could see no difference in the immediate or delayed effect in DNA and RNA synthesis. In this tissue the uptake of ^{32}P into the RNA is somewhat decreased by irradiation. At the lower irradiation doses of 1650 r and 1500 r there is a suggestion that the decrease of RNA synthesis is no longer found in the "resistant" strain and there is, perhaps, a chance that we

may be able to demonstrate a slight strain difference here. At 1350 r there is very little effect on RNA or DNA synthesis in either strain.

It remains to be proved that the radioresistance still persists in the Dittrich strain, and experiments are in progress to test this point. At present, it seems that the effects on DNA synthesis have very little to do with the radiosensitivity of the tumours. These experiments are being repeated on new strains sent from Germany.

REFERENCES

ABERCROMBIE, M., and HARKNESS, R. D. (1951). *Proc. roy. Soc. B.*, **138**, 544.
ABRAMS, R. (1951). *Arch. Biochem.*, **30**, 90.
BENNETT, L., KELLY, L., and KRUECKEL, B. (1954). *Fed. Proc.*, **13**, 181.
DITTRICH, W., HÖHNE, G., and SCHUBERT, G. (1956). *In* Progress in Radiobiology p. 381. Edinburgh: Oliver & Boyd.
GURSCH, F. N., VARS, H. M., and RARDIN, I. S. (1948). *Amer. J. Physiol.*, **152**, 11.
KELLY, L. S. (1952). *Proc. Soc. exp. Biol., N.Y.*, **81**, 698.
KELLY, L. S. (1953). *Acta radiol., Stockh.*, Suppl. 116.
KELLY, L. S. (1954). *Proc. Amer. Ass. Cancer Res.*, **1**, 24.
KELLY, L. S., HIRSCH, D., BEACH, G., and PAGE, A. H. (1955). *Radiation Res.*, **2**, 490.
KELLY, L. S., and PAYNE, A. H. (1953). *Fed. Proc.*, **12**, 76.
LUDWIG, S. (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 158.
MEE, L. (1956). *In* Progress in Radiobiology p. 12. Edinburgh: Oliver & Boyd.
ORD, M. G., and STOCKEN, L. A. (1956). *Biochem. J.*, **63**, 3.
PELC, S. R., and HOWARD, A. (1933). *Acta radiol., Stockh.*, Suppl. 116.
PRICE, J. M., and LAIRD, A. K. (1953). *Cancer Res.*, **10**, 650.
SCHWARTZ, H. C., and BARKER, S. B. (1954). *Fed. Proc.*, **13**, 131.
STERN, I., and SHAPIRO, B. (1953). *J. clin. Path.*, **6**, 158.
STOWELL, R. (1949). *Arch. Path.*, **46**, 164.

DISCUSSION

Koller: I cannot add anything more to Dr. Holmes' data except to emphasize the fact that after irradiation there are chromosome breaks in dividing cells of the regenerating liver. I may mention, however, the very interesting fact that the number of mitoses which appear in regenerating liver, treated with colchicine, is extremely high. We found that 28–33 hours after hepatectomy, 85 per cent of the cells are undergoing mitosis. It seems that division must be extremely rapid and that the duration of the mitosis is very short.

Holmes: I didn't mention the colchicine experiments. We put the colchicine in 3 hours before killing the animal because we had the strong conviction that over a long period it was rather poisonous and decreased the rate of mitosis. That certainly does not happen when it is only in for 3 hours. As regards the induction of radiation injuries, I think I am right in saying that usually one does not get many chromosome breaks if one irradiates cells of *Vicia* or *Tradescantia* early in interphase before DNA synthesis, but that was not true in the case of regenerating liver. Many breaks were found when irradiation with 450 r was given before the beginning of the period of DNA synthesis, i.e. at 12 hours after hepatectomy and 13 or 15 hours before mitosis would be expected. This would normally be described as irradiation during interphase.

Koller: Yes, that was the case.

Howard: How long was the colchicine present?

Holmes: Only the 3 hours. The ^{32}P was also injected 3 hours before killing; we tried to collect all the mitoses that happened in that period. There are no colchicine counts shown in Fig. 1.

Lajtha: This was surgical hepatectomy?

Holmes: Yes, ours was surgical hepatectomy.

Lajtha: The difference between your results and those of Dr. Kelly may be due to the different means of producing hepatectomy. She produces it by chemical means, and that does not destroy the nuclei completely. Perhaps she has an experimental condition similar to that which Dr. Forssberg has, with some DNA present in dying cells which may serve as a pool for the regenerating cells.

Holmes: That is a very interesting suggestion.

Gray: Do I understand correctly that the wave of mitosis is not delayed if you irradiate just before hepatectomy, but that it is if you irradiate after hepatectomy?

Holmes: No, if you irradiate just before there is just as much delay as if you irradiate in the "sensitive" period. If you irradiate it 24 hours before hepatectomy there is some recovery. The rate of DNA synthesis is low and some chromosome breakage is seen, but some mitoses do appear at 27 hours.

de Hevesy: Do you know the actual growth rate, in the course of $1\frac{1}{2}$ hours, of those tumours in your last table? What percentage would that be? They can't grow without formation of DNA.

Holmes: I do not know.

de Hevesy: The ratio of the specific activity of DNAP at the end of your experiment and the mean value of the specific activity of cellular orthophosphate P during your experiment is supposed to give the percentage of DNA molecules formed in the course of $1\frac{1}{2}$ hours. If you follow up the growth of your turnover for a longer time and extrapolate from these data the amount of DNA formed during $1\frac{1}{2}$ hours you will presumably find half of the value supplied by the radioactive data only. We and others interpreted such findings in the old days as indicating that with the formation of two new DNA goes hand in hand the disappearance of one. Recent work carried out by you and others indicate however that no appreciable amount of DNA molecules disappears in

the course of growth. A possible explanation of the above-mentioned discrepancy is that the orthophosphate we isolate from the tumour is an artifact and has a lower specific activity than that utilized in the building up of the DNA molecule. If the genuine orthophosphate has about twice the specific activity of the isolated one, the above-mentioned discrepancy clearly disappears. We must furthermore consider the possibility that the precursor of DNAP is not orthophosphate P but the phosphorus of a compound formed in the cell boundary under participation of highly active extracellular P. In experiments carried out on composite homogenates made up from isolated nuclei and isolated cytoplasm fractions from rabbit liver tissue Davidson observed recently that the acid-soluble fraction of the cell sap contains a more effective precursor of DNA than inorganic phosphate.

Holmes: In one particular series of regenerating livers Dr. Richards found that a number of cells had formed an amount of DNA which brought the content up to an octoploid level. If many cells synthesize this large quantity before they divide, the earlier calculations of the amount of new DNA required for each mitosis must be altered.

It has been found that the octoploid content of DNA is the usual content in the Ehrlich ascites tumour cell immediately before mitosis (Kelly, L. S., and Jones, H. B. (1956). *Fed. Proc.*, **15**, 108). Furthermore, the same has been found to be true of the Krebs ascites tumour cells (Richards, B. M., Walker, P. M. B., and Deeley, E. M. (1956). *Ann. N.Y. Acad. Sci.*, **63**, 831).

THE INDUCTION OF CHROMOSOMAL ABERRATIONS BY IONIZING RADIATIONS AND CHEMICAL MUTAGENS*

C. P. Swanson

Department of Biology, The Johns Hopkins University, Baltimore

AND

Bengt Kihlman†

Institute of Physiological Botany, University of Uppsala

A DECADE ago, when the late D. E. Lea's (1946) book on radiobiology appeared, a physical explanation of the events leading to the production of chromosomal aberrations seemed eminently satisfactory. The dosage and intensity relationships, the results of fractionation experiments, the spacing of ions along known paths by different types of radiations, and the ideas revolving around the target theory and the "breakage-first" concept, fitted together sufficiently well to give a good measure of confidence in a strictly physical interpretation of the available data. Seven years later, however, it was possible to state that "The main features of the biological experiments (with ionizing radiations) make very good sense when viewed from the standpoint of radiation chemistry" (Gray, 1953). As it applied to aberrations induced by ionizing radiations, this enlarged concept—for it was an expansion of earlier ideas rather than a shift in perspective—stemmed from the initial studies of Thoday and Read (1947, 1949) and their

* Acknowledgement of financial support of the work reported here is gratefully made to the U.S. Atomic Energy Commission [Contract AT (30–1) 1695] and to the National Science Foundation (Research Grant NSF–G2233). We wish also to acknowledge our indebtedness to Dr. A. V. Beatty for permission to cite from his unpublished studies.

† On leave of absence from the University of Uppsala to The Johns Hopkins University under the Exchange Visitor Programme of the U.S. Information and Educational Act of 1948.

later extension by Giles and his co-workers (reviewed by Giles, 1954) which demonstrated the central rôle which oxygen plays in governing the degree of chromosomal damage. The radiochemical aspects of radiation and their relation to chromosomal studies have been adequately treated elsewhere, and it needs only to be recalled that the effects of radiation on biological systems can be modified by a variety of experimental conditions such that the chromosomal damage may be amplified or diminished. Any initial complacency generated by the knowledge of radiochemical events was of short duration, however, and it is now evident that the radiochemical events are but a link which, in the living cell, connect the physical events of radiation with the observable effects such as aberrations. Latarjet and Gray (1954) have expressed this in the following way:

I		II		III		IV
Absorption of radiant energy	→	Primary radiochemical reactions	→	Chemical reaction chains	→	Observable lesions

Step III constitutes the greatest unknown in the above chain of events, and is the one on which our attention will be largely focused. The inadequacy of the first two steps to account for all of the parameters encountered in chromosomal studies with ionizing radiations has been made evident by a variety of observations: among others, the fact that oxygen alone is capable of inducing aberrations (Conger and Fairchild, 1952), the discovery of differential rates of breakage and rejoinability during the course of cell division (Sparrow and Maldawer, 1950; Deschner and Sparrow, 1955) and the rôle of metabolic inhibitors in modifying the final frequency of aberrations (King, Schneiderman, and Sax, 1952; Wolff and Luippold, 1955). These latter studies strongly suggest the involvement of oxidative metabolism in the ultimate extent and expression of radiation damage.

Although the radiobiological experiments may make "good

sense" when viewed from the point of view of radiochemistry, certain pieces of data indicate that the radiochemical events in the cell can be magnified without appreciably altering the final frequency of aberrations. Gray, on several occasions (1953, 1954*a* and *b*), has pointed out that there exists a close parallel between the number of ion pairs per unit of path length and H_2O_2 production on the one hand and the frequency of aberrations on the other. It is a well known fact, however, that the frequency of aberrations is proportional to the oxygen concentration only at low levels; when the concentration of oxygen rises above 20 per cent little increase in aberration frequency is found even though H_2O_2 production continues to increase. Allen (1954) and Ebert (1955) have also shown that the addition of small amounts of hydrogen to oxygenated water leads to a striking increase in H_2O_2 production, presumably by promoting the reaction: $2\ HO_2 + H_2 \rightarrow 2\ H_2O_2$. If it is presumed that the cell behaves as an aqueous system, then increases in aberration frequency are to be expected when the cells are exposed to oxygen-hydrogen mixtures. Mr. T. Merz has carried out these experiments in our laboratory, using a variety of hydrogen-oxygen mixtures, and finds that while in *Tradescantia* microspore chromosomes chromatid deletions are somewhat increased whenever hydrogen is added to oxygen, exchanges and isochromatid deletions remain relatively unaffected. Dominant lethals in *Drosophila* are also greater in a mixture of 20 per cent O_2 : 80 per cent H_2 than they are in air, but there is no obvious relationship which suggests that the amount of radiation damage is proportional to H_2O_2 production. These results are also supported by the data of Kimball (1955) which indicate that H_2O_2 is not involved in the induction of genetic damage in *Paramecium*. There is danger, of course, in forcing too close a comparison between what is known to occur in oxygenated water and what is expected to occur in a cell which would be buffered against environmental change. Several interpretations are possible here, but more important is the fact that the data force us to look beyond the radiochemical events for

some of the answers, and it is obvious that the complications are many.

The studies of Wolff and Luippold (1955; see also Wolff and Atwood, 1954) have been particularly instructive in focusing attention on the involvement of metabolic systems in the final expression of radiation damage in terms of chromosomal aberrations. Wolff and his co-workers have demonstrated, as have others before him, that breakage is oxygen-dependent, but in addition his data also support the idea that the time period of rejoining is similarly governed by the amount of cellular oxygen. The older arguments concerned with the "breakage versus restitution" controversy can now be dispensed with since both are shown to be oxygen-dependent. Based on fractionation techniques, and the use of inhibitors of oxidative metabolism, these studies have been interpreted as a demonstration of the fact that the rejoining of broken ends is an energy-requiring event, and Wolff has proposed that the radiation injures the metabolic system upon which the repair of broken chromosomes depends. The greater the dose of radiation, the greater the damage to this system, and the longer the delay before rejoining can take place. The breaks initially induced remain open during this period. Treatment of root-tip cells between radiations with low temperatures, KCN, CO in the dark, and DNP inhibit the rejoining system, and so prolong the period between breakage and rejoining; ATP, but not AMP, shorten the period. The rôle of externally applied ATP in effecting the rejoining system must remain questionable for the time being since it is unlikely that it penetrates the cell to act as such. However, the fact that it is the time period of rejoining that is affected rather than the rejoinability of broken ends itself is shown by providing the cells with these same agents as a posttreatment after only a single dose of radiation is given. The final frequency of aberrations induced by single doses of radiation remains unaffected by any posttreatment.

Wolff's conclusions are supported by the data of Beatty, Beatty and Collins (1956). Using a total dose of 400 r of X-

rays, and with intensities ranging from 1 to 50 r/minute, it has been shown that the frequency of chromosome interchanges increases as the intensity decreases when exposures were carried out in the absence of oxygen (helium was used to replace the oxygen of the cell). The reverse trend, of course, holds for irradiations in air or in oxygen. The frequency of aberrations obtained with an intensity of 1 r/minute in helium was approximately equal to that found at an intensity of 25 r/minute in pure oxygen. These results are somewhat unexpected, and following the line of reasoning expressed by Wolff and Luippold (1955), it would appear that quite different sets of conditions prevail in the oxygen as opposed to the helium series. Radiation at a comparatively high intensity in oxygen leads to a high rate of breakage, but this is offset by the fact that a large proportion of the breaks restitute during the period of exposure; high frequencies of aberrations are obtained in the low intensity helium series with a considerably lower frequency of breaks but with negligible rejoinability during the period of radiation. It has logically been assumed by Beatty, Beatty and Collins (1956), in the 1 r/minute helium experiments, that the circumstances of anoxia are greatly exaggerated by a continued depletion of residual oxygen through respiration during the 400 minutes of exposure to radiation. The energy sources of the cell which might otherwise be available for rejoining purposes would be sharply depressed by removal of the oxygen, and all breaks induced would remain open and be available for rejoining when oxygen was once again added to the cell. This hypothesis can be tested further. If cells are pretreated for 400 minutes in an oxygen-free atmosphere, and then irradiated at various intensities, they should have their energy reserves at a low ebb, rejoining should not take place, and no intensity effect should be observed. Or, conversely, pretreatments in an oxygen-free atmosphere for varying periods of time, followed by radiation at a constant intensity, should yield frequencies of aberrations which increase as the duration of the pretreatment increases. The latter experiment

has been carried out (Beatty, Beatty and Collins, 1956), and the data are in conformity with expectations.

The results discussed above deal generally with the rejoining process, and shed little or no light on the breakage of chromosomes by radiations. Except for centrifugation and infrared radiation, breakage appears to be unmodifiable by posttreatments, and there is no experimental evidence which suggests that breakage by radiation is mediated through the normal metabolic pathways of the cell. It is of interest here to recall that there is a lack of intensity effect in the mature sperm of *Drosophila* (Muller, 1940) and in the generative cells of dehydrated pollen grains of *Tradescantia* (J. C. Kirby-Smith, unpublished). These are cells in which the chromatin is densely packed, and where it is unlikely that metabolic systems having a high yield of free energy would be operative to provide the necessary requirements for rejoining. Metabolic activity in these cells would increase with the uptake of water which occurs at the time of fertilization for *Drosophila* sperm and during the germination of *Tradescantia* pollen, and the opportunities for rejoining would become available. The fact that a reduction in aberrations in these two types of cells is obtained when irradiation is carried out in the relative absence of oxygen is added support for the concept that breakage itself is determined only by the physical and radiochemical events in the Latarjet-Gray reaction chain rather than by the succeeding metabolic events (see later).

Before attempting to fit the above data into a model system it will be well to consider, in a comparative way, the effects of chemical mutagens on aberration induction. A large variety of chemicals which differ appreciably in structure and reactivity can induce chromosomal aberrations, but it seems desirable to confine our attention to some of those which have been studied intensively. These are di(2: 3-epoxypropyl) ether (DEPE), 8-ethoxycaffeine (EOC), and maleic hydrazide (MH). *Vicia faba* root-tips have been used as experimental material. It is impossible as yet to say how any of these chemicals affect chromatin to bring about the induction of aberrations, but it

is apparent that their actions differ among themselves, and that all differ in their action from the ionizing radiations. Chemically, MH is more similar to EOC than it is to DEPE. Both MH and EOC are relatively unreactive compounds whereas DEPE has a high chemical reactivity. The biological effect of MH or EOC may or may not be related to the fact that both possess a structural resemblance to the bases in nucleic acids: EOC is a purine derivative, and MH, as first realized by Loveless, is a structural isomer of uracil. Of the three mutagens discussed, MH is the only one having acidic properties. This is reflected in the strong pH dependence of this compound for effectiveness; it is a powerful mutagen at a pH of 5, but at the concentrations used its effect diminishes rapidly at higher pH levels, and practically vanishes at a pH of 7 or above. These facts suggest that the unionized compound penetrates much faster than the ionized form.

In spite of their structural similarity, the biological effect of MH bears no closer resemblance to that of EOC than it does to that of DEPE. Both MH and DEPE act in early interphase with the first aberrations appearing between 8 and 12 hours after treatment (Revell, 1953; McLeish, 1953). Aberrations induced by EOC appear within 2 hours after treatment, indicating that it, like X-rays, acts in late interphase or early prophase (Kihlman, 1955). MH and DEPE also show the same relationship to temperatures; in both cases the effectiveness of the compound increases rapidly with increasing temperature. The effectiveness of EOC increases in the range of temperatures from 0° to 10° C, but then decreases at higher temperatures. Few aberrations are obtained with EOC at temperatures above 25° C.

On the other hand, the biological effectiveness of EOC and MH, but not that of DEPE, is greatly reduced when oxidative phosphorylation is inhibited during the period of treatment. Their differences are also emphasized when one considers their specificity of action which is reflected in a non-random distribution of aberrations. In *Vicia* root-tip cells, DEPE

produces a heavy concentration of aberrations in heterochromatic segments in the middle of the long arm of S-chromosomes (Loveless and Revell, 1949). MH-induced breaks are also localized in heterochromatin, but in this instance, the heterochromatin lying close to the centromere in the nucleolar arm of the L-chromosomes is most frequently involved (McLeish, 1953). EOC confines its major effect to the same arm, but the aberrations involve principally the nucleolar constriction (Kihlman and Levan, 1951). The distribution of breaks is more strikingly non-random the lower the concentration of the mutagen, indicating that there are preferential sites of action which may become obscured, however, if the concentration of mutagen is increased.

Two other observations are of interest in this respect. The localization of breaks differs also with time after treatment as well as with concentration of mutagen. DEPE seems to induce a lowered percentage of localized breaks at 48 hours after treatment than it does at 24 hours, while EOC-induced breaks are more sharply localized at 22 hours than they are either earlier (6 hours) or later (48 hours). What these data mean in terms of a mechanism of action is not entirely clear. The suggestion has often been made that chemicals and ionizing radiations induce breaks in interphase chromosomes because of their interference with DNA synthesis. No critical evidence bears on this point, but the differential times and sites of action, as well as the differential effectiveness of mutagens and radiations under conditions of anoxia and in the presence of inhibitors of oxidative metabolism, would indicate that an induced disturbance of DNA synthesis cannot provide a complete and satisfactory answer as to their mode of action. The seeming correspondence of many of these results with those obtained by Wolff on the rejoining process is striking, and suggests a common metabolic pathway somewhere in the chain of events leading to the observable aberrations induced by ionizing radiations and chemical mutagens, but it remains to be determined whether these common pathways are at comparable stages in the chain of events.

Discussion

An attempt can be made to formalize our notions of interactions at the chromosomal and physiochemical levels within the cell during and after exposure to ionizing radiation or chemical mutagens. At the chromosomal level the two major categories of events are breakage and rejoining, but each is subdivisible. As to the breakage category, it is unlikely that all breaks consist of fully broken chromatids or chromosomes. Although difficult to assess, the concept of potential as distinguished from primary breakage (Thoday, 1953) gains some credence from the infrared studies done in these laboratories. Probably the ion density of the radiation would be the principal factor involved in determining the spectrum of chromosomal damage (Swanson, 1955*a*), i.e., the greater the ion density the greater the portion of primary breaks as opposed to potential breaks, and it seem likely, although no proof is at hand, that the oxygen level of the cell would also be a contributing factor (Swanson, 1955*b*). The rejoining system can be operationally divided into restitution and recombination, these being competitive actions for the disposal of broken ends of chromosomes in the nucleus. The great majority of broken ends do not, of course, contribute to observable aberrations, but if we adhere to the idea of the existence of potential breaks then a reduction in the number of damaged sites could come about through the repair of potential breaks or the restitution of primary breaks. There is no possibility of distinguishing between them at present.

The experimental facts permit us to modify the Latarjet-Gray scheme in the following way:

I		II		III		IV		V
Physical events	→	Primary radio-chemical events	→	Secondary radio-chemical events	→	Metabolic events	→	Observable effects

The distinction between stages II and III is made on the basis of time, with II covering the short-lived radicals and III

those which may persist for some time. The studies of Wolff and Luippold (1955) and Beatty, Beatty and Collins (1956) with ionizing radiations and those of Kihlman (1955, and unpublished) with chemical mutagens make stage IV a necessary part of the chain of events. There is no need here to discuss the physical and radiochemical aspects of this system since they have been adequately covered by Gray elsewhere (1953; 1954*a* and *b*), but it is of interest to assess our present knowledge of the relationship between the Latarjet-Gray chain of events and those taking place at the chromosomal level. Stages I–III are obviously related to breakage, but to weigh the contribution of each stage to breakage would require that one determine the relative importance of direct versus indirect effects of ionizing radiations. At present it would appear that we can only state that the indirect effects seem to outweigh the direct effects if only because we realize that a small amount of radiant energy introduced into a cell can lead to an inordinate amount of damage. No direct and convincing evidence is yet at hand which permits us to state that metabolic systems in the cell have anything to do with the breakage of chromosomes by ionizing radiations. It is true, of course, that the sensitivity of the chromosomes changes with the stages of cell division, but this variable sensitivity may well be due to the state of the chromosome rather than to any metabolic system which contributes directly or indirectly to breakage. However, the fact that spontaneous breakage is a variable phenomenon from species to species, and often within species, suggests that it may be due to altered physiological conditions such as those brought on by nutritional deficiencies (Steffensen, 1953). On the other hand, it seems quite certain that the metabolism of the cell governs the action of certain chemical mutagens. Thus, as indicated earlier, MH and EOC are both dependent upon the oxygen of the cell for effective action, and the effects of both are suppressed by inhibitors of oxidative phosphorylation. The chemical mutagens, to be sure, would not involve stages I–III in acting on chromosomes, but in their place it is prob-

able that chemical (non-enzymatic) and biochemical (enzymatic) events of comparable importance can be substituted. It would appear, for example, that the initial action of DEPE is chemical in nature since it is highly reactive, its effectiveness is governed by the law of mass action (Revell, 1953), and its action is not modified by pretreatment with DNP (Loveless, 1953). EOC and MH, on the other hand, are modified in their effectiveness by agents which inhibit oxidative phosphorylation, and it is likely that their initial action is through enzymatically controlled steps in cellular metabolism.

Wolff's data indicate that the restitution and recombination of ends of chromosomes broken by events taking place in stages I–III require energy supplied through oxidative metabolism, and that the rejoining system is comparatively sensitive to small amounts of radiant energy. Considerable protection is afforded it by anoxic conditions. It is suggested, as a consequence, that the principal damage to the rejoining system is inflicted by active radicals and their derivatives which have been induced by radiation. It is further tempting to see a connection between the results described above which indicate a possible rôle of oxidative phosphorylation in the production of chromosomal aberrations and the recent findings of Allfrey, Mirsky and Osawa (1955) which point to the oxidative generation of energy-rich phosphate within the nucleus, a process heretofore considered to be confined to the mitochondria. It would appear, therefore, that the nucleus does not necessarily exist and function in an anaerobic environment as suggested by Stern (1955), but much remains to be done before the links in the chain are connected.

It has not been possible to separate breakage and rejoining following exposure to chemical mutagens as Wolff seems to have done in his radiation experiments. One cannot, therefore, fully assess their effects on either system other than to point out that with some chemicals oxidative phosphorylation obviously determines their mutagenic effectiveness or ineffectiveness. With other chemicals such a point of view cannot be expressed with the same degree of surety. It is our

impression, however, that oxidative phosphorylation is always involved in some of the steps in the chain of events leading to chromosomal aberrations. The experimental data so far available suggest that this step is toward the end of the chain, and that it may be one normally occurring in the chromosome. The metabolic events (stage IV) are probably several in number, and the larger the number before the actual induction of aberrations the less obvious will be the influence of inhibitors of oxidative metabolism on chemical mutagenesis.

Admittedly we are treading on somewhat uncertain ground in the above discussions. We have, for example, spoken of breakage and rejoining as separable events in the induction of chromosomal aberrations. This may or may not be so. At the present time, the radiation experiments seem most satisfactorily explained by making this distinction, but both Revell (1953) and Loveless (1953), although differing in their interpretations, agree in considering these two events inseparable so far as the induction of aberrations by chemicals is concerned. The chemical experiments described above have not yielded critical evidence on this point and further discussion of them will be reserved for subsequent papers where a more detailed examination of the data can be made.

REFERENCES

ALLEN, A. O. (1954). *Radiation Res.*, **1**, 85.
ALLFREY, V. G., MIRSKY, A. E., and OSAWA, S. (1955). *Nature, Lond.*, **176**, 1042.
BEATTY, A. V., BEATTY, J. W., and COLLINS, C. (1956). *Amer. J. Bot.*, **43**, 328.
CONGER, A. D., and FAIRCHILD, L. M. (1952). *Proc. nat. Acad. Sci., Wash.*, **38**, 289.
DESCHNER, E., and SPARROW, A. H. (1955). *Genetics*, **40**, 460.
EBERT, M. (1955). Radiobiology Symposium (Liège), p. 30. New York: Academic Press.
GILES, N. H. (1954). Radiation Biology, Vol. 1, part 2, p. 713. New York: McGraw Hill.
GRAY, L. H. (1953). *Brit. J. Radiol.*, **26**, 609.
GRAY, L. H. (1954*a*). *Radiation Res.*, **1**, 189.
GRAY, L. H. (1954*b*). *Acta Radiol.*, **41**, 63.
KIHLMAN, B. (1955). *Hereditas, Lund*, **41**, 384.
KIHLMAN, B., and LEVAN, A. (1951). *Hereditas, Lund*, **37**, 382.

KIMBALL, R. F. (1955). *Ann. N.Y. Acad. Sci.*, **59**, 638.
KING, E. D., SCHNEIDERMAN, H. A., and SAX, K. (1952). *Proc. nat. Acad. Sci., Wash.*, **38**, 34.
LATARJET, R., and GRAY, L. H. (1954). *Acta Radiol.*, **41**, 61.
LEA, D. E. (1946). Actions of Radiations on Living Cells. Cambridge University Press.
LOVELESS, A. (1953). *Heredity* (Suppl.), **6**, 293.
LOVELESS, A., and REVELL, S. (1949). *Nature, Lond.*, **164**, 938.
MCLEISH, J. (1953). *Heredity* (Suppl.), **6**, 125.
MULLER, H. J. (1940). *J. Genet.*, **40**, 1.
REVELL, S. H. (1953). *Heredity* (Suppl.), **6**, 107.
SPARROW, A. H., and MALDAWER, M. (1950). *Proc. nat. Acad. Sci., Wash.*, **36**, 636.
STEFFENSEN, D. (1953). *Proc. nat. Acad. Sci., Wash.*, **39**, 613.
STERN, H. (1955). *Science*, **121**, 144.
SWANSON, C. P. (1955*a*). *Genetics*, **40**, 193.
SWANSON, C. P. (1955*b*). *J. cell. comp. Physiol.*, **45**, 285.
THODAY, J. M. (1953). *Heredity* (Suppl.), **6**, 299.
THODAY, J. M., and READ, J. (1947). *Nature, Lond.*, **160**, 608.
THODAY, J. M., and READ, J. (1949). *Nature, Lond.*, **163**, 133.
WOLFF, S., and ATWOOD, K. C. (1954). *Proc. nat. Acad., Sci., Wash.*, **40**, 187.
WOLFF, S., and LUIPPOLD, H. E. (1955). *Science*, **122**, 231.

DISCUSSION

Hollaender: I wonder whether the effect of oxygen alone would produce as many breaks in *Tradescantia* as a metabolic system?

Swanson: This is one reason for assuming that a metabolic system is involved. With Conger's work on oxygen alone it seems to me that it is much more likely that a metabolic system is involved than it is to relate the effect to hydrogen peroxide or some reactive system of that sort. One can, for instance, follow Ebert's lead in adding hydrogen to an oxygenated system which, if we consider the cell to be an aqueous medium, would lead to an increase in hydrogen peroxide concentration. This will induce a larger proportion of breaks, but our data bear no simple relationship to Ebert's curve. Added hydrogen increases the frequency of aberrations. There must be some saturation phenomenon involved; we have gone through the whole range of hydrogen-oxygen mixtures, but we just get a slight increase and it doesn't matter what the hydrogen oxygen mixture is.

Alper: In fact Ebert's curve for hydrogen peroxide yield with this mixture is very flat, particularly for low doses.

Swanson: Then I have misinterpreted Ebert's curves because I assumed from them that a mixture of 25 per cent hydrogen and 75 per cent oxygen gave a very large increase in hydrogen peroxide formation.

Alper: The hydrogen peroxide determinations were made on the basis of total dose. If you irradiate water in the presence of oxygen alone,

without hydrogen, you get a family of curves for hydrogen peroxide yield against oxygen concentration. With low doses you get the equilibrium yield at low oxygen concentrations, whereas with a high dose you don't get the equilibrium yield until the oxygen concentration is high. If you start with 100 per cent oxygen, then add hydrogen, the equilibrium yield rises sharply, for low doses, and is independent of hydrogen concentration over a wide range of hydrogen-oxygen mixtures. As you decrease the oxygen to zero and increase hydrogen to 100 per cent the yield stays constant and falls sharply to zero as oxygen concentration falls to zero. With higher doses the range of mixtures over which you get constant yield becomes much smaller, and the relative proportions of hydrogen and oxygen become critical. With low doses, however, you get a very wide range of mixtures with which yield is unaltered.

Swanson: What do you mean by low doses? What range?

Alper: 10–20,000 r with the methods he has used up to now, but presumably this range would be extended if one could use lower doses.

Swanson: In the hundreds?

Alper: It depends on how low he has gone with the oxygen. I don't know what the actual range was. This doesn't mean that hydrogen peroxide is constant.

Howard: Prof. Swanson, you say that if you add hydrogen to an oxygenated system the frequency of aberrations is increased. This is different to what we found with bean roots in which we got less damage in presence of hydrogen, even when oxygen was also present.

Swanson: I am aware of that. The study done by Mr. Merz was on the microspores of *Tradescantia*. I think that the gases were accurately controlled. There is some danger, of course, of a flash-back, but we managed to avoid it. We got practically the same results regardless of what we added in the way of hydrogen, making certain at all times that the oxygen was at least 20 per cent.

Howard: Did you have hydrogen under pressure?

Swanson: No, this was just the flowing gas, under no pressure.

Haddow: Am I right in believing that maleic hydrazide has no effect on animal cells? I think maleic hydrazide certainly has no effect whatever of these kinds on animal cells.

Swanson: I only know that McLeish reported that it was ineffective on onion chromosomes where no visible heterochromatin is demonstrable, but this is not entirely correct because it will break them very effectively if you adjust the temperature and the pH. Maleic hydrazide, therefore, is not a mutagen restricted in its activity to heterochromatin, although that is the preferred site of action.

Haddow: We have come across this in a practical way in connection with maleic hydrazide which, it was believed, might possibly be carcinogenic. But it is certainly not a strong carcinogen.

Swanson: I wonder whether this would be true if the data were taken over a period of time, as with the diepoxide. We assumed that there was no oxygen effect with the diepoxides, but if one examines the cells at a later time after treatment, at least with the metabolic inhibitors, one does observe a decided effect.

Koller: I wish to mention that Dr. Auerbach would like to repeat her early experiments, because she does not think that an oxygen effect in the case of mustards really exists. The experimental conditions were not sufficiently stringent.

Spiegelman: Do these *Tradescantia* microspores which you treat have any metabolism which is detectable? Do they respire or glycolize?

Swanson: Tradescantia is treated in the bud stage, and it is going through very active division, so I presume it is an actively metabolizing cell.

Alper: You quoted some data where after seven hours exposure to oxygen you had 73 breaks/100 cells at a dose rate of 1 r/min. How many would that be with just seven hours of oxygen?

Swanson: These data are from a paper by Beatty, Beatty and Collins (*Amer. J. Botany*, in press). They graciously loaned me the data for this symposium. There are no control data, however, which disturbed us a little, and that is why we have become interested in the problem. Root-tip cells seemed to be the easiest material for us to study at the moment and we obtained a rather high frequency of aberration with long periods of anoxia alone.

Howard: Is it possible that in your work on *Vicia* root-tips, where you use all these different treatments—the temperature treatment, blockage of the metabolism of the cell, the chemicals you are using, and radiation—you can really take into account the fact that you must be altering the mitotic cycle, and therefore the moment at which you look at the treatment is highly relevant? Would you not need rather complete time-curves to control this?

Swanson: I expect that you are right here. The only thing I can say is that it is the conviction of Dr. Kihlman, who has done the chemical mutagen work, that this is not a factor. He had worked this out in terms of time relationships and he feels that the mitotic activity is not a controlling factor, at least not to the extent that it throws the data all out of proportion.

Koller: On one of your slides you showed two curves, one concerning the interchanges and the other the isochromatid breaks. You pointed out that irradiation and chemical mutagens interfere with the chromosome rejoining process which has two components, restitution and recombination. Did you find a decrease in the interchanges and a similar decrease in the isochromatid or in the open breaks?

Swanson: With the mutagens there are relatively few breaks that do not undergo recombination. We don't find many incomplete exchanges or the non-sister reunion type of isochromatid deletions. The proportions of the two do not change with treatment, although they do change with time. This, of course, is a function of the spatial relationships of the chromatids, but there is no appreciable change in the proportions of the two types with any particular treatment. So I think that they are comparable types of aberration.

Van Bekkum: With regard to the metabolic inhibitors such as dinitrophenol, have you any data on the effect of ATP, for instance?

Swanson: I can quote only the data of Wolff, to the effect that ATP

externally applied, shortens the rejoining period. He also has unpublished data which indicate that adenine, added to the solution in which the roots are immersed, does it even more rapidly.

Brachet: Is there any cytological effect of the inhibitors alone, dinitrophenol for instance?

Swanson: They don't appreciably affect the mitotic cycle or the appearance of the cells at the concentrations and times employed.

Brachet: In *Acetabularia*, where the nucleolus is very conspicuous, it changes quickly and considerably in shape and RNA content in the presence of dinitrophenol and similar substances. Regarding the part of the cell where these poisons may be acting, it is interesting that, in *Acetabularia*, Stich got the same transformation as with dinitrophenol by leaving the algae in the dark for several weeks. Of course, since the chloroplasts are only present in the cytoplasm, the effect is primarily the reaction of the nucleolus to metabolic events taking place in the cytoplasm. In eggs also, dinitrophenol produces big cytological changes and mitotic inhibition; but it seems that it induces disturbances of the nuclear RNA, rather than changes in the chromosomes.

Swanson: We have looked at them when stained with Feulgen and there appears to be no difference between them.

Pirie: You said that dinitrophenol reduced this effect of mutagenic chemicals. Yet it is not a blocking agent to metabolism, it is an upsetting agent, an uncoupling agent which is surely very different to blocking; respiration will increase, and your ATP will go down.

Swanson: It should be emphasized that we have only reached the point of asking biochemical questions. With EOC, for example, the toxic effects are not interfered with by dinitrophenol; if the temperature is raised up 28°, the major action of EOC is one of reducing root growth. The roots actually turn black. This is not interfered with by dinitrophenol, so that the mutagenic effect on the chromosomes must be quite different.

Holmes: Did Kihlman find any change in mitotic index with dinitrophenol?

Swanson: There is no change in mitotic index with dinitrophenol. This has been checked very carefully.

PRIMARY SITES OF ENERGY DEPOSITION ASSOCIATED WITH RADIOBIOLOGICAL LESIONS

L. H. Gray

British Empire Cancer Campaign Research Unit in Radiobiology, Mount Vernon Hospital, Northwood

The concern of this symposium is with metabolic pathways in the development of radiobiological damage. Direct observation of the metabolic activity of the irradiated cell may hit the trail early or late. It may possibly be a help in interpreting observations of metabolic activity to consider whether there is any independent evidence as to where any of the pathways begin. The evidence might in principle be either chemical or anatomical.

In the study of biological damage induced by u.v. radiation, useful information has been obtained by comparing "action spectra" with absorption spectra. Mutation, chromosome fragmentation, inhibition of colony formation, are among the effects which are usually associated with the nucleotide type of absorption, while spheration of the nucleolus and spindle damage are among those in which the primary energy absorption appears to be in protein. The study of biological damage induced by u.v. radiation and radiomimetics, however, gives us no reliable evidence concerning damage induced by ionizing radiations, since many cases are known in which the damage induced by these several agents proceeds, at least in part, by different pathways (Gray, 1954).

Ionizing radiation delivers energy to atoms in a highly localized, but unselective, manner, almost regardless of the molecular configurations of which they form a part. The types of chemical change which follow in small and large molecules have been described by Dale and Butler (this

symposium), but direct observation of the living cell immediately after irradiation has not so far yielded any definitive information as to the relative importance of these changes for the initiation of radiobiological damage. Burns (1954) has set limits of $-1{\cdot}7$ cc./mole to $+2{\cdot}4$ cc./mole to the early chemical changes associated with lethality in haploid yeast, which are probably recessive lethal mutations. This figure may be compared, for example, with changes of -20 to -70 cc./mole for the volume change associated with each peptide bond broken in the enzymic hydrolysis of protein. Moreover, positive results were obtained by McElroy (1952) and McElroy and Swanson (1951) for u.v. and nitrogen mustard mutations in *Neurospora* and *Aspergillus*. The interpretation of the negative results with ionizing radiation should, however, be accepted with caution as far as reactions which take place along the track of the ionizing particle are concerned, since they involve the application of thermodynamic considerations to a highly transient and irreversible system.

The yield of information by the chemical approach is thus very small. It may be that we have not yet examined radiation response in chemical systems at the right level of organization for the important physicochemical changes to be revealed.

Anatomical Evidence

The anatomical approach has proved more profitable. A variety of experimental techniques have been brought to bear on this problem.

1. The use of radiations of limited penetrating power

Many years ago Zirkle (1932) took advantage of the limited range of the polonium alpha particle (up to 30 μ) and of the fact that in the fern spore the nucleus (diameter 10 μ) lies to one side of the protoplast (diameter 38 μ), to compare the results of irradiations which included or excluded the nucleus. The biological criteria were inhibition of cell division. All these effects could be brought about by irradiations from the

side remote from the nucleus, but only by the use of doses at least twenty times as great as when the nucleus was included in the field of irradiation. Zirkle drew attention to the interesting fact that the cracking of the cell wall, which is a function related to water imbibition and to chlorophyll development, which might not normally be thought of as under nuclear control, was evidently initiated by moderate doses of radiation through an injury originating in the nucleus.

About the same time Henshaw and Henshaw (1933) exposed *Drosophila* eggs to polonium alpha particles at different times after the eggs were laid and found a strong positive correlation between the proportion of eggs prevented from hatching by a given exposure to alpha radiation and the inclusion of nuclei in the irradiation field. The correlation was the more striking because the stage of development which brought the largest number of nuclei into the field of irradiation happened to be one of minimum sensitivity to X-rays, which, of course, irradiate the whole egg uniformly.

Within recent years this type of study has been extended by Pollard (1955) and his colleagues to smaller cells by the use of very slow electrons. In this case specimens have to be irradiated *in vacuo*. This automatically excludes any form of radiation damage which may proceed from ionization of the aqueous phase and does not measure the inactivating effect of radiation under physiological conditions. On the other hand, the fact that the Yale Group have obtained results with biologically active molecules and viruses under precisely these conditions, which in general accord rather well with studies of the same molecules and viruses irradiated under more natural conditions, may be considered to justify a cautious acceptance of the results obtained with slow electrons. These indicate in the case of *Bacillus subtilis* spores (Hutchinson, 1955) that from the standpoint of viability (colony-forming ability) after irradiation, the spore has a completely insensitive coat about 230 Å thick, a body of intermediate sensitivity which is of smaller size but approaches to within 20–30 Å units of the surface at one point, and a comparatively sensitive core.

The structure of *B. subtilis* has not yet been established by staining methods but in *Bacillus megatherium* cytological structure has been described by several workers (Robinow, 1953; Yuasa, 1956) which corresponds rather strikingly to Hutchinson's (1955) description.

Davis (1954) has studied the inactivation of T1 bacteriophage by slow electrons. She finds that the surface coat of the phage particle, about 100 Å units thick, is extremely insensitive. If the surface coat is identified with protein in this case and the core with DNA, then it is evident that when the whole virus is exposed to radiation in the dry state the inactivation is predominantly associated with energy deposited in the DNA. Energy deposition in the protein only brings about inactivation at a dose level sixteen times higher than that which suffices when energy is absorbed in the DNA. The irradiation of oriented tobacco mosaic virus particles leads to the same conclusion (Pollard and Whitmore, 1955).

2. Micro Beams

The irradiation of selected small regions of living cells by protons and by pencils of u.v. radiation has been brought to a high degree of technical perfection by Zirkle and Bloom (1953). In the case of the proton beam 80 per cent of the particles fall within a circle 2·5 μ in diameter and 96 per cent within a circle 5 μ in diameter. The convergent heterochromatic u.v. beam intensely irradiates an approximately isodiametric volume about 7 μ across. The cells principally studied have been from adult newt heart cultured at 23° C and observed by phase contrast illumination as they enter and pass through division.

Irradiation of the centromere region of a chromosome by either radiation may result in the loss by that chromosome of directed movement, so that it never moves to the equatorial plate. Similar irradiation of regions of the chromosome not including the centromere were never observed to produce this effect. Somewhat larger exposure to u.v. resulted in a change in refractive index which was at first confined to the length of

the chromosome actually irradiated but which spread in the course of 30 minutes to three times the original length. This effect was only produced by protons when the dose was increased more than 100-fold. Micro-beam irradiation of prophase chromosomes with a few dozen protons was found to be very effective in producing chromosome stickiness, and chromosomes lacking functional centromeres and chromosome fragments. Irradiation of metaphase chromosomes was about as effective in producing chromosome stickiness but very rarely destroyed the function of the centromere or produced chromosome fragments. In contrast to the marked effects of a few dozen protons delivered to small chromosomal regions, relatively huge numbers were ineffective when delivered to similarly small extrachromosomal regions. Thousands were delivered to ends of spindles, and hundreds of thousands to cytoplasm. No effects could be seen at the irradiation sites or elsewhere. An occasional sticky chromosome which was seen could be ascribed to protons which were scattered into the chromosomes when very large numbers were aimed at extrachromosomal targets.

In contrast to the protons, heretochromatic u.v. radiation produced very striking effects when extrachromosomal regions were given exposures of the same order as those which, given to chromosomal regions, produced stickiness and loss of centromere function. To destroy the spindle and induce a deranged metaphase it is not necessary to include part of the spindle in the u.v. irradiated region. A slightly greater exposure of an equal volume of cytoplasm produces the same effect, from which it was concluded that effects on the spindle were quite probably due entirely to the absorption of u.v. radiation by some cytoplasmic component. We may recall here the observations of Carlson and McMaster (1951), who used a variety of monochromatic u.v. radiations, that in grasshopper neuroblasts the derangement of spindle mechanism exhibited a protein-like action spectrum.

It had, of course, long been believed that the chromosome fragments seen at metaphase following the irradiation of

many different types of cell in interphase or early prophase were the result of the passage of a single ionizing particle either through, or in the immediate vicinity of, the chromosome thread.

Zirkle and Bloom's experiments strongly support this view but they do not, of course, prove that only one particle was involved. They only irradiated a very small fraction of the length of the chromosome thread in any one exposure, and in order to secure a reasonable frequency of breakage had to use 10–12 protons, corresponding to an average dose within the micro beam of perhaps 2,000 rads. The inference that breaks are produced by single ionizing particles still rests on the linear relation between breakage frequency and dose. Since this relation was observed by Kotval and Gray (1947) to hold even at such low doses (4 rads.) that few of the irradiated nuclei were traversed at any time by more than one particle, there can be little doubt about the validity of this inference in this particular case. Similar considerations make it rather certain that the induction of lysogenicity in bacteria observed by Marcovich (1954) must be due to a single electron.

3. Nuclear Transfers

The fertilization of the ovum provides a natural means of introducing an irradiated nucleus into unirradiated cytoplasm and, in special cases, of the opportunity to study the converse, namely the development of a cell containing an unirradiated nucleus in irradiated cytoplasm.

As remarked earlier (p. 257) developing *Drosophila* eggs are more readily killed by nuclear than by cytoplasmic damage. Opinion is still divided as to whether the killing of these eggs at their most radiosensitive stage can reasonably be ascribed entirely to nuclear damage.*

Direct evidence, however, has been provided by Whiting (1949) through her studies of radiation damage in *Habrobracon*.

* *Note added in proof:* Rather conclusive evidence in favour of the importance of nuclear damage at this stage has been presented by Ulrich (1955), who compared the lethality among eggs in which either the anterior half only or the posterior half only had been exposed to X-rays.

By appropriate matings, individuals could be obtained derived either from irradiated cytoplasm and irradiated (female) nucleus or irradiated cytoplasm and unirradiated (male) nucleus. The dose required to inhibit development of the latter was 54,000 rads., which was twenty-two times as great as that required to inhibit the former. The author concluded that in those animals which failed to develop after irradiation of the cytoplasm only, the injured cytoplasm acts in a direct manner in killing the embryo and not indirectly through injury to the untreated chromosomes. However, Nakao (1953) has recently presented evidence to show that when fairly heavily irradiated silkworm eggs are fertilized by unirradiated sperm within $2\frac{1}{2}$ hours of irradiation, the eggs which are laid show plenotypic changes which are characteristic of the loss or mutation of certain genes located on the paternal chromosomes.

One of the earliest observable effects of radiation in many types of cell is what is commonly referred to as the inhibition of mitosis or of cell division. In eggs it is observed as a delay in first cleavage, in yeast as a delay in the second budding after irradiation, and in dividing tissues as an immediate fall in the mitotic index. Most, or all, cells of a population which is at a uniform stage of development are affected, and to an extent which increases with the dose. It is measurable in grasshopper neuroblasts after a dose of only 4 r, in many plant and animal dividing tissues after doses of about 50 r, in yeast after about 1,000 r and in sea urchin eggs after about 10,000 r.

Careful investigation of the phenomenon in several of the classes of cell mentioned has revealed, as a common feature, a prolongation of the time taken by the cell to pass from the terminal stage of interphase to the end of prophase. The classical experiments of Henshaw (1940) on cleavage delay in the eggs of the sea urchin (*Arbacia punctilata*), discussed quantitatively in detail by Lea (1946), have provided the following information:

(a) Irradiation at the dose levels employed does not affect fertilization, the approach of the two pronuclei, or

fusion, all of which proceed at the normal rate, but it does result in a great prolongation of the first mitotic prophase.

(b) Irradiation of either sperm or egg alone before fertilization produces a comparable delay in cleavage.

(c) The injury to the egg is one which is repaired at a rate of about 1 per cent/minute at 20–25° C and 0·3 per cent/minute at 0° C. No detectable repair takes place in the sperm.

(d) Irradiation of enucleated eggs prior to fertilization causes no cleavage delay, though the time taken in cleavage is in this case much longer than in the cleavage of a normal egg.

(e) When the sperm and the egg are each irradiated prior to fertilization, the injuries sustained by each exactly summate in their effect on cleavage delay, if allowance is made for repair.

The conclusion of Henshaw and of Lea that in this case the prolongation of prophase is the result of a reparable injury sustained by nuclear material seems inescapable. In view of the period of the cell cycle which is critical in this phenomenon, it is suggested that the injury is one connected with the condensation of the chromosomes. The further conclusion that the nuclear material is directly injured is much less well founded. Direct injury of nuclear material seems probable since the delay is the same whether it is the sperm or the egg which is irradiated despite the enormous disparity in the respective volumes of cytoplasm and because of the absence of effect in enucleated eggs. However, the sperm is not devoid of cytoplasm, and nuclear injury could conceivably arise through a disturbance in cytoplasmic metabolism. The dose relations are not linear but those of an effect which varies with the log of the dose. This suggests the existence of material, essential to the passage of the cell through the critical phase, which is inactivated at a rate which is some function of the administered dose and repaired at a rate which depends on

the general level of metabolic activity (*cf.* the "cumulative dose" concept developed by Lea (1946), Friedenwald and Sigelman's (1953) treatment of mitotic delay in corneal epithelium and Burns' (1954) treatment of division delay in yeast).

Temporary arrest of cells about to enter division may be caused by a great many agents, including hypoxia, depletion of phosphate and glycogen. The possibility—indeed one may even say the probability—must always be kept in mind that a given result is achieved by different pathways under the influence of chemical, u.v. and ionizing agents.

In one or two materials the transfer of nuclei by microdissection procedures has been used to study the respective rôles of nuclear and cytoplasmic damage. Duryee (1949) studied the incidence of nuclear pyknosis which develops in the course of a few days at 22° C in salamander oocytes after exposure to doses of around 3,000 rads. The oocytes were in early meiosis at the time of irradiation. This form of damage appears within half an hour if the dose to the oocytes is raised to 50,000 rads. When nuclei were isolated from the egg by microdissection and washed free of cytoplasm, this dose of radiation produced negligible damage. Since in the intact egg this dose did not produce immediate visible damage it is clear that even after such relatively large doses metabolic processes must intervene before the injury is apparent, and it is therefore not unexpected that isolated nuclei appear unaffected. That the nuclei have not been rendered insensitive by the microdissection was shown by the fact that the injury developed if they were exposed to irradiation in the presence of cytoplasmic brei, prepared by grinding in a mortar the contents of three eggs from which the nuclei had been removed. The brei could either have provided conditions essential for the metabolism of the nucleus or injured the nucleus indirectly through a toxic product formed in the brei as a result of irradiation. Evidence in support of the latter view was contributed by microdissection experiments in which cytoplasm from irradiated eggs (3,000 rads.) was transferred to unirradiated eggs and resulted in nuclear pyknosis. The microdissection

procedure itself occasionally resulted in pyknosis. Discrete chromosome fragmentation, which arises from irradiation of the intact oocyte, was not recorded as a result of the injection of irradiated cytoplasm.

Ord and co-workers (1952) have used nuclear transfers between amoebae to evaluate the site of action of nitrogen mustard and X-radiation when these agents give rise to division delay and cell death. It was not possible to irradiate isolated nuclei. Inferences were based on comparisons between amoebae treated whole and amoebae reconstituted from an untreated nucleus and cytoplasm treated either in the presence or absence of a nucleus. Although both agents are lethal to the cell at considerably lower doses when the nucleus is treated than when only cytoplasm is treated, important differences between the effects of the two agents were noted. With regard to radiation damage, it was found that damage to the cytoplasm was of two kinds, a reversible damage which is maximal at 100,000 rads., and lethal damage which becomes prominent at 290,000 rads. This is, of course, a very high dose. Exposure of an aqueous solution to this dose could completely transform reactants at the millimolar concentration level. It is not surprising that it should be lethal to cytoplasm. The comparatively high mean lethal dose for nuclear damage (120,000 rads.) may be due to the occurrence of each genetic factor at a high multiplicity (high polyploidy or polyteny) as is almost certainly the case with *Paramecium*, which has a radiosensitivity comparable with that of *Amoeba* (Kimball, 1949).

It was concluded that a dose of X-rays which is lethal to all nuclei in the amoeba will not cause lethal damage to cytoplasm, and that the nuclei were probably damaged independently of the cytoplasm.

Polyploid and Multinucleate Cells

The study of polyploid and multinucleate micro-organisms has thrown considerable light on the matter under discussion.

The beautiful experiments of Latarjet and Ephrussi (1949) with haploid and diploid yeast have become classical in this connection. Within the last few years our knowledge of the radiobiology of yeast has been greatly extended by the very careful investigations of the Berkeley Group led by Tobias (1952). Haploid yeast cells exposed to X-rays either form a double, of which only one cell contains nuclear material, or form a complete colony. Irradiated diploid cells give rise to all intermediate forms between the double and the complete colony. Correspondingly haploid cells which had not budded at the time of irradiation show a strictly exponential relation between survival fraction and dose, whether exposed to X- or alpha-radiation, while diploid cells show a sigmoid relation for both radiations, indicating that more than one particle is responsible for the lethal damage. Budding cells, in which mitosis is in progress, are much more resistant than the interphase cells and even in haploid yeast do not show an exponential relation between survival and dose.

An elaborate analysis of the radiosensitivity of haploid, diploid, triploid and tetraploid cells, and of the sensitivity of clones grown from survivors from a previous irradiation, led the authors at one time to conclude that the lethality was entirely explicable in terms of recessive lethal mutations. This proved to be too simple an interpretation. At present, death of haploid cells is ascribed to the induction of recessive lethal mutations—some of which could be chromosomal—and that of other ploidies to a mixture of recessive and dominant lethality. Contributions from cytoplasmic damage cannot be excluded in the case of the higher ploidies.

Finally, we may consider the very interesting experiments of Atwood (1955, and personal communication), who studied the radiosensitivity of binucleate and multinucleate *Neurospora* conidia. Binucleate conidia were formed in which the two nuclei carried, as markers, genes for nutritional deficiencies. Such binucleate cells will grow on minimal medium. When irradiated and grown on complete medium, sigmoid survival curves were observed similar to those for diploid yeast, the

shape corresponding well with expectation for a two-hit type of effect. When grown on minimal medium, however, log survival curves were strictly linear and with the slope approximately double that of the asymptotic (low survival) portion of the log survival curves of organisms grown on complete medium ($LD_{37} \sim 15{,}000$ r). These relations are to be expected between survival on complete and minimal medium if the injury is of the nature of a dominant lethal mutation, since on minimal medium inactivation of either nucleus would be lethal to the spore. Atwood concluded that the injury which was lethal to the spore was entirely nuclear in origin and of the nature of a dominant lethal mutation. The experiment was repeated using different combinations of the markers and with trinucleate cells, each of which was doubly marked. All were consistent with the hypothesis of dominant lethal mutation as the cause of death. Whatever the precise nature of the damage to the genetic material, its effects are obviously very far-reaching since a nucleus so damaged is unable even to synthesize the single amino acid or other growth factor required by the second nucleus. It is remarkable that metabolic activity can be so completely inhibited by the passage of a single ionizing particle.

Summary

The results which have been discussed are summarily classified in Table I. It is at once obvious that the forms of damage which have been analysed are not representative of radiobiology as a whole. Seven out of sixteen entries are concerned with cell reproduction, and in these the criteria adopted are tantamount to a test of the reproductive integrity of the cell. Six other cases are concerned with nuclear components or nuclear function. To destroy the reproductive integrity of the cancer cell is the aim of radiotherapy and in this connection the effects listed in Table I are of special interest despite their limited range. The cell is a unit of biological organization and, as might be expected, vital

Table I

SITES OF ACTION OF IONIZING RADIATION

	Material	*Doses in rads.*	*Criterion of effect*			*Provisional assignment of site of primary injury*		*Possibility of mechanical loss of genetic material*	*Mitosis intervenes*	
			Nuclear		*Not known to involve the nucleus*	*Nuclear*	*Cytoplasm*			
			Synthesis of gen. material essential	*Synthesis not necessarily essential*						
Virus	Tl (dry)		Rep. integ. (Plaques)			DNA (0·95)	Protein (0·05)		+	Davis (1954)
Micro-organisms	Haploid yeast	15,000	Rep. integ.			(1·00)	(~0)	−	+	Burns (1954)
	Diploid yeast	60,000	Rep. integ.			(~1·00)	(~0)	+	+	Latarjet and Ephrussi (1949)
	Neurospora conidia	15,000	Rep. integ.			(~1·00)	(~0)	+	+	Atwood (personal communication)
Protozoa	*Amoeba*	100,000	Rep. integ.			~0·6	~0·4	−		Ord *et al.* (1952)
Crustacea	*Arbacia* sperm	10,000		Cleavage delay		~1·00	~0		−	Henshaw (1940)
Amphibia	Salamander oocytes	3,000		Nuclear pyknosis		+	+	−	−	Duryee (1949)
Insect	Silkworm eggs	3,000		Egg colour		+	+	−	−	Nakao (1953)
	Dros. eggs	200	Rep. integ. (Hatching)			+	?	+	+	Henshaw and Henshaw (1933)
	Habrabracon eggs	2,000	Rep. integ.			>0·95	<0·05	+	+	Whiting (1949)
Plant	Fern spores	10,000 (α)		Cell division		>0·95	<0·05	−	−	Zirkle (1932)
	,, ,,	30,000 (α)			Chlorophyll synthesis	>0·95	<0·05	−	−	
	,, ,,	80,000 (α)			Cracking of cell wall	>0·95	<0·05	−	−	
In vitro Culture	Newt hart			Chromosome breaks		~1·0	~0	−	−	Zirkle and Bloom (1953)
	,, ,,			Chromosome stickiness		~1·0	~0	−	−	
	,, ,,			Inactivation of centromere		~1·0	~0	−	−	
	,, ,,			Spindle damage						
Mammal	Human erythrocyte	30,000			Net loss of potassium (40,000 r)	0	1·0	−	−	Sheppard and Stewart (1952)

processes can be destroyed by a sufficiently large dose to either nucleus or cytoplasm. The mammalian erythrocyte provides positive evidence for cytoplasmic damage (Sheppard and Stewart, 1952). In ten of the cases listed in Table I it has been found, however, that over a certain range of doses a given effect is produced if the nucleus is included in the field of irradiation and not produced if the nucleus is either excluded from the field of irradiation or introduced into the cell after irradiation of the cytoplasm. In one or two cases the same effect has been achieved by the irradiation of cytoplasm without irradiation of nuclear material, but only by the use of doses 3–20 times greater than those which are sufficient when nuclear material is irradiated. If we admit also the less direct evidence presented in the case of lethality in haploid and diploid yeast and in *Neurospora* conidia, then in thirteen cases out of sixteen it may be said that the cell owes its sensitivity predominantly to the susceptibility of its nuclear material to injury by ionizing radiation.

Account must be taken of the fact that in all the cases considered, the nucleus has been irradiated in the presence of cytoplasm. It might be argued, therefore, that the injury which makes itself apparent in the nucleus is in fact secondary to a cytoplasmic injury.

It would appear that nuclear damage can be subdivided into:

(a) Forms in which the damage is seen in most or all cells at a given stage of development, is graded with dose, and for which formal dose relations are not characteristic of a single particle initiation. Cleavage delay, division delay and unspecific nuclear pyknosis are typical examples.

(b) Effects seen in some cells and not at all in others, such as the induction of mutations and chromosome structural damage, having dose relations characteristic of individual particle effects.

With regard to Class (b) it would, in my view, be difficult to sustain this hypothesis in the light of the experiments of

Zirkle and Bloom and of the relations which have been observed between survival and dose.

The evidence in the case of Class (a) damage is equivocal. Some of the experiments of Henshaw and Whiting were expressly designed to reveal nuclear injury resulting from primary damage to the cytoplasm, but failed to do so even at quite high dose levels. However, nuclear damage arising from a transient disturbance in cytoplasmic metabolism or to labile toxic products produced in the cytoplasm could have escaped detection, despite the fact that eggs were fertilized immediately after irradiation.

Kaufmann, McDonald and Bernstein (1955) have observed that doses of 250–1,000 r delivered to *Drosophila* larvae salivary gland cells *in vivo* affect the colloidal properties of the cells, and that doses of 1,000 r delivered *in vitro* affect the stability of gels of calf thymus nuclei. Somewhat analogous experiments have been made by Anderson (personal communication). Kaufmann and co-workers show further that nucleoprotein is damaged by doses (1,000 r) which are without effect on either the DNA or the protein components when irradiated separately. These experiments appear to have an important bearing on Class (a) nuclear damage. They may also provide the clue to the outstanding sensitivity of lymphocytes and thymocytes since the lethal effects of radiation on these two classes of cell bear certain resemblances to biological effects known to proceed from Class (a) nuclear damage.

The metabolic study of Class (b) nuclear damage, as induced by ionizing radiations, appears to pose an extremely difficult problem on account of the random nature of the primary injuries. It would seem that there must be a period of time during which a lethal injury is following hundreds of different pathways in different cells. Mutations which ultimately prove lethal may only be expressed a considerable time after irradiation, either because the definitive mutation is delayed until the time of gene reproduction or because nuclear control over cellular metabolism is not immediate. During this interval of time an opportunity is offered for the study of metabolic

disturbances induced at higher dose levels as a result of either nuclear or cytoplasmic damage, but these may be quite irrelevant to the metabolic pathways leading to loss of reproductive integrity at low dose levels.

REFERENCES

ATWOOD, K. C. (1955). *Amer. Naturalist*, **88**, 295.
BURNS, V. W. (1954). U.C.R.L. Report 2812.
CARLSON, J. G., and MCMASTER, R. D. (1951). *Exp. Cell Res.*, **2**, 434.
DAVIS, M. (1954). *Physiol. Rev.*, **94**, 293.
DURYEE, W. R. (1949). *J. nat. Cancer Inst.*, **10**, 3.
FRIEDENWALD, J. S., and SIGELMAN, S. (1953). *Exp. Cell Res.*, **4**, 1.
GRAY, L. H. (1954). *Radiation Res.*, **1**, 189.
HENSHAW, P. S. (1940). *Amer. J. Roentgenol.*, **43**, 899.
HENSHAW, P. S., and HENSHAW, C. T. (1933). *Biol. Bull., Woods Hole*, **64**, 348.
HUTCHINSON, F. (1955). *Ann. N.Y. Acad. Sci.*, **59**, 494.
KAUFMANN, B. P., MCDONALD, M. R., and BERNSTEIN, M. H. (1955). *Ann. N.Y. Acad. Sci.*, **59**, 553.
KIMBALL, R. F. (1949). *Genetics*, **34**, 412.
KOTVAL, J. P., and GRAY, L. H. (1947). *J. Genet.*, **48**, 135.
LATARJET, R., and EPHRUSSI, B. (1949). *C.R. Acad. Sci., Paris*, **229**, 306.
LEA, D. E. (1946). Actions of Radiations on Living Cells. Cambridge University Press.
MARCOVICH, H. (1954). *Nature, Lond.*, **174**, 796.
MCELROY, W. D. (1952). *Science*, **115**, 623.
MCELROY, W. D., and SWANSON, C. P. (1951). *Quart. Rev. Biol.*, **26**, 348.
NAKAO, Y. (1953). *Nature, Lond.*, **172**, 625.
ORD, M. G., DANIELLI, J. F., HARRIS, E. B., and LAMERTON, L. F. (1952). *Nature, Lond.*, **170**, 921.
POLLARD, E. C. (1955). *Ann. N.Y. Acad. Sci.*, **59**, 469.
POLLARD, E. C., and WHITMORE, G. F. (1955). *Science*, **122**, 335.
ROBINOW, C. F. (1953). *J. Bact.*, **66**, 302.
SHEPPARD, C. W., and STEWART, N. (1952). *J. cell. comp. Physiol.*, **39**, Suppl. 2.
TOBIAS, C. A. (1952). Symposium on Radiobiology, p. 357. London: Chapman & Hall.
ULRICH, H. (1955). *Naturwissenschaften*, **16**, 468.
WHITING, A. R. (1949). *Biol. Bull., Woods Hole*, **97**, 210.
YUASA, A. (1956). *Nature, Lond.*, **177**, 387.
ZIRKLE, R. E. (1932). *J. cell. comp. Physiol.*, **2**, 251.
ZIRKLE, R. E., and BLOOM, W. (1953). *Science*, **117**, No. 3045, 487.

DISCUSSION

Latarjet: I should like to say a few words about this problem of ploidy and survival curves. The main fact, as Dr. Gray mentioned, is that the number of hits which comes out of the survival curve fits with

the ploidy. This has been found in all types of cells which have been investigated so far. However, in spite of the fact that several full papers have been written by others on this subject, a major difficulty remains which Dr. B. Ephrussi and I encountered in our original work. When ploidy increases, not only does the number of hits increase accordingly, but also the radioresistance of the individual unit which undergoes the hit. The slope of the straight part of the survival curve decreases.

Let us consider the simplest situation, that, for example, of several virus particles inside the same cell, which can multiply within the irradiated cell as long as they are themselves active, and let us suppose that the cell remains infective as long as it contains one active particle. It has been found that the survival curve of the "infectivity" of such multiply infected cells, early after infection, i.e. before the virus has begun to multiply, fits in with the multiplicity of infection. The curve agrees with the general equation:

$$\text{survival} = 1 - (1 - e^{-\alpha D})^n$$

where n is the number of infecting particles, D the dose, and α the coefficient which characterizes the radiosensitivity of the individual particle. When n increases, α does not change, all curves have parallel straight parts.

In the case of ploidy, however, α decreases when n increases. At the same time, the morphology of the lesions may differ. In haploid yeast, one observes only immediate death and double giant lethals. In polyploid yeast, one observes in addition the many classical figures of delayed recovery. I think that in the latter, some kind of multiplicity reactivation takes place, but I really do not know what this means for chromosomes. As a matter of fact, no simple hypothesis has given a satisfactory account for the experimental results.

Gray: With regard to Norman and Atwood's results, I think these are exactly the results to be expected because in a cell containing two nuclei with nutritional deficiency you have two targets, the inactivation of either of which is lethal if you plate on minimal medium. If you plate on complete medium you must inactivate each of two targets. The slope of the former inactivation curve being twice that of the latter is thus in accordance with expectation. In fact, the slopes differ by a little less than a factor of two, because there were recessive lethals to be taken into consideration. I had not spotted this in the yeast.

Swanson: In *Tradescantia* one can irradiate cells which are either haploid or diploid, and the chromosome sensitivity is different on a chromosome basis. Yet these presumably are the same types of chromosomes. We have done this fairly extensively on diploid and tetraploid individuals of the same species of *Tradescantia*, but we found a considerable difference in sensitivity in microspore chromosomes.

Butler: Dr. Gray mentioned the effect of pressure; I don't think that really works. The thermodynamic theory is based on equilibrium considerations, there is an activated state through which the material is passing. With ionizing particles you have got something equivalent

to a very high temperature. If you wanted to work this out you would have to introduce the equivalent temperature, in other words the temperature which would produce that particle, so that your temperature would be very high and you would not expect any volume change. The same applies to the effect of temperature. There should be no effect of temperature on the initiating step, but if you do get an effect of temperature it is an effect on the subsequent steps.

Lajtha: Does one get these ploidy effects only if one irradiates under dry conditions, or do they occur if the organism is suspended in dilute medium?

Gray: They are ordinary living cells, they are not dry.

Lajtha: The opposite happens in mammalian cells and in living bean roots where the actual synthetic stage during which the 2 *n* DNA increases to 4 *n* seems to be less sensitive to small doses of radiation than the interphase stage with its stationary 2 *n* amount of DNA.

Gray: If you score the amount of chromosome damage per cell, this amount of damage increases with ploidy, but of course it may be less lethal to the cell because damage to any one chromosome is more likely to be covered by the other chromosome sets. Diploid yeast is less sensitive than the haploid; the same applies to *Neurospora*, the higher ploidy is less sensitive despite the fact that more actual chromosomal damage is produced.

Koller: Miss Lamy, working with Muller in Edinburgh, found no difference in radiation sensitivity between the diploid and the triploid *Drosophila*.

Gray: This was concluded in yeast in the higher ploidies also.

Alexander: I think an important point arises both out of Dr. Gray's and Dr. Swanson's papers and that is: is the breakage of the chromosomes already a metabolic event or is this the primary chemical change following directly on the absorption of the energy from the radiation? I believe that the amount of energy which Lee and others have calculated as necessary for giving a break is not sufficient to turn the nucleoprotein from the chromosome from a gel into a sol. This would have to be the case if the chromosome-break was produced by chemical action. I think even for the break it may be necessary to postulate some metabolic process. This view, I believe, is supported by the experiments on the effect of radiation on the properties of the nucleoprotein gel which I mentioned earlier (this symposium, p. 57). Irradiation of whole cells produces much greater changes than irradiation of nuclei. A possible interpretation is that the nucleoprotein is not damaged by a few hundred r at the concentration at which it is present in the cell, but that an enzyme acting on the nucleoprotein is released by radiation. Dounce showed that there are enzymes in the cell which can liquify this nucleoprotein gel extremely efficiently, and that only the most careful preparations of nuclei which are absolutely clean from all adhering matter can give nucleoproteins which remain as stable gels.

Swanson: If you are referring to chromosome breaks, wouldn't this presuppose that there should be a posttreatment effect?

Alexander: Yes, but it might have to be extremely rapid. In this

connection I was encouraged by the report from Dr. Hollaender (this symposium p. 206) that mitotic suppression could be reduced by treatment with hypertonic salt immediately after irradiation. A posttreatment would have to be given quickly because if it is in fact a question of allowing enzyme to get as a gel particle, then this may happen within seconds after irradiation. The suggestion that the attack on the chromosomes does not follow directly from the uptake of energy certainly raises the possibility that mutations may be prevented by a suitable aftertreatment.

Swanson: In terms of breaks we have been able to use only two devices to modify their frequency by posttreatment: one is mechanical, i.e. centrifugation, the other is infrared, which is another irradiation. There is no other means of which I am aware.

Alexander: One really wants a physical method, because chemicals would have to diffuse in and it is obviously difficult for one diffusion process to catch up with another one.

Gray: Dr. Alexander, I wonder whether, in the experiments to which you refer, the number of nuclei which are affected is proportional to dose, or whether all nuclei are affected but to a degree which varies with the dose.

Alexander: I suspect that radiation affects many nuclei to some extent, the effect is not confined to a few. These experiments are rather difficult to do accurately because of the difficulty of isolating clean nuclei. Some of the apparent discrepancies in the literature concerning the effect of radiation on cellular nucleoproteins could be explained if a metabolic process intervened since time factors then become all-important.

Butler: I think that would be explained if the nuclear damage caused by radiation made the nucleoprotein more open to attack by enzymes. Nucleoprotein preparations all slowly break down, and it appears to us that that is due to the action of deoxyribonuclease, because we found that the breakdown is accompanied by a degradation of the DNA. If you examine the DNA from an intact gel and then examine the DNA from the nucleoprotein gel which has been kept for some time, and has become liquified, you find that it is the DNA that has become degraded. Therefore we conclude that this is an action of deoxyribonuclease which may well have come from the cytoplasm, so that your observation could be explained as the action of radiation on the nucleus causing some degree of damage to the nucleoprotein material which in some way renders the entry of the deoxyribonuclease easier.

Alexander: That was the exact interpretation which I had placed on it, that is, the irradiation is not sufficient to liquify the gel. All it did was to enable a metabolic process to take place. Butler's suggestion that the nucleoproteins from cells become more susceptible to attack by enzymes after irradiation has already been established by Cole and Ellis. But this effect by itself could not explain the formation of chromosome breaks since any enzymes capable of attacking nucleoproteins must be stored well away from the chromosomes if cells are to survive at all.

Butler: There would have to be initial radiation damage. You are merely showing it up.

Pirie: Does your weakening effect increase with time after radiation or can you stop it, i.e. if you irradiate your whole cell and immediately take out the nuclei, are they then stable?

Alexander: There is certainly a time effect with whole cells but for technical reasons it is very difficult to reduce the time interval to less than about five minutes.

Holmes: Do we know any useful DNAse inhibitors? You could not apply them after irradiation, they would not act quickly enough. Could they be used as protective substances? In the case of a proteolytic enzyme, for instance, we might imagine that cystine and cysteine would interfere with it.

Alexander: This is an idea to which Prof. Bacq and I have given much thought. We don't think that DNAse is the only enzyme concerned and it is possible that there are some other enzymes which are capable of de-geling the nucleoprotein in other ways.

Spiegelman: Has the effect of citrate been tried on this?

Alexander: Yes, but citrate does not protect.

Gray: Where is the DNAse situated in the cells?

Brachet: It is mostly cytoplasmic.

Alexander: Is there evidence for any DNAse in the nucleus?

Brachet: There is always the possibility that some of the DNAse is sticking to the nuclei. One finds a little DNAse in them, but it is difficult to say, when an enzyme is present only in small amounts in a given cellular fraction, that it has not been adsorbed during the isolation processes.

Hollaender: We would like very much to have a physical anti-ionization agent. Hypertonic salt solution is probably something of a physical agent; putting a new balance of ions in where this balance has been upset. But if one could visualize an agent which would counteract the ions, hold them in some form immediately following radiation, I think the problem would be very much simpler.

Swanson: There is one possibility in the infrared. Some work has been published recently by Moh and Withrow (1955, *Plant Physiol.*, abstracts), where the 6200 Å region is believed to be inhibitory in terms of chromosome breaks, while the 7100–8200 Å region adds to the X-ray damage. These correspond to the regions of the spectrum that were worked on by Hendricks in his seed germination studies, with the shorter wavelengths being inhibitory, the longer ones capable of breaking dormancy.

EFFECTS OF RADIATION AND PEROXIDES ON VIRAL AND BACTERIAL FUNCTIONS LINKED TO DNA SPECIFICITY

RAYMOND LATARJET
Laboratoire Pasteur du l'Institut du Radium, Paris

IN the course of the last few years, attention has been drawn to the production of organic peroxides within irradiated living cells, tissues, and organisms. These substances are in general very reactive, and, in the presence of oxygen, they can elicit chain reactions of peroxidation. It has been thought that they might play the rôle of intermediates in the production of certain radiolesions, i.e., the rôle of true radiomimetics, profoundly affecting cell metabolism. As a matter of fact, peroxides have already been considered responsible for the posteffect of radiation, and for the sensitizing influence of oxygen; mutagenic and lethal effects have been obtained with these substances; conversely, certain radiolesions have been prevented by posttreatment with peroxidases.

These considerations led me some time ago to undertake quantitative experimental comparisons of some effects produced by organic peroxides and by radiation in simple biological systems such as a bacterial transforming agent, bacteriophages and bacteria, with special emphasis on some specific hereditary characters carried by their DNA. The present paper groups the first results obtained by my collaborators and myself. It should be clearly understood:

(a) that these results are of preliminary character;
(b) that differences among biological systems and even among peroxides forbid any generalization on these results at the present time;
(c) that, in our opinion, peroxides are only some examples (perhaps very important ones) among the many chemical mediators which are brought into play in irradiated living systems.

Material and Methods

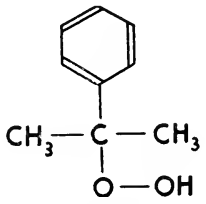
1. *Bacteria and bacteriophages.* *Escherichia coli*, non-lysogenic strain B, and the phages of the T series, lysogenic strain K12, its temperate phage λ, and the strain K12S sensitive to λ, were used according to the classical techniques for growth and plaque formation.

2. *The transforming agent TP Sr*, which confers resistance in *Pneumococcus* to 2 mg. of streptomycin per ml. without inducing bacteria of intermediate resistance, was chosen. TP of several stocks were used, containing about 0·6 mg. of DNA per ml. The techniques for preparation and purification of this nucleic acid, for producing the bacterial transformations, and for quantitative titration of the active agent have already been described in detail (Ephrussi-Taylor and Latarjet, 1955).

3. *The X-ray source* was my usual molybdenum target tube operating at 37 kv and up to 42 ma. Its radiation, filtered through 0·05 mm. aluminium, delivered up to 1 krad./sec. to the preparation, with an average wave-length of 0·9 Å. The samples were irradiated in plexiglas cups containing 0·4 ml. spread in a layer which absorbed about 10 per cent of the incident radiation. In some experiments, the cups were placed in vacuum chambers with aluminium windows.

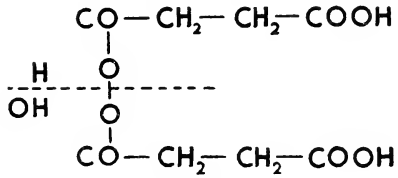
4. *Two organic peroxides* have been used:

(i) Commercial cumene hydroperoxide (Hercules Powder Co.), a viscous liquid which contained 40 per cent of active product (Formula I). Its aqueous solution reached saturation for a concentration by weight of about 10^{-4}.



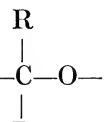
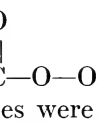
Formula I

(ii) Crystallized disuccinoyl monoperoxide (Formula II), synthesized in this laboratory by R. Royer and B. Ekert. This is in the form of prismatic colourless crystals, which melt at 128° C, and explode weakly and without danger in a flame. They are water-soluble at a concentration by weight of 10^{-2}, and are very stable in the dark and in dry air. This compound is immediately hydrolysed when dissolved in water, yielding one molecule of succinic acid and one molecule of succinic peracid, which is in fact the active principle of the solution.



Formula II

The solution, in the dark at room temperature, liberates about 5 per cent of its peroxidic oxygen per day. All solutions were titrated for peroxide activity by Mr. B. Ekert. The cumene peroxide was titrated by the thiofluorescein method (Dubouloz, Monge-Hedde and Fondarai, 1947); the succinic peroxide was titrated by oxidation of ferrous iron and spectrophotometric dosage (302 mμ) of the ferric iron. Precision reached 2 μg. of peroxide per ml.

In some respects, the  function of the cumene compound differs from the  of the succinic compound. While many similarities were observed, striking differences sometimes appeared in their action on the biological samples. Cumene peroxide does not render the solution acid,

but succinic peroxide introduces some acidity which must be taken into account by suitable controls in certain experiments. If, in order to keep the pH at a given value, one dilutes the peroxide in an acetate buffer, the titre in peroxidic oxygen, and consequently the activity, remains unchanged. However, we did confirm that the results which are reported in this paper were actually due to the chemical effect of the peroxide, not to acidity.

Inactivation of bacteria by peroxide

Bacteria were washed, then resuspended in saline in the presence of peroxide. At given times, aliquots were diluted

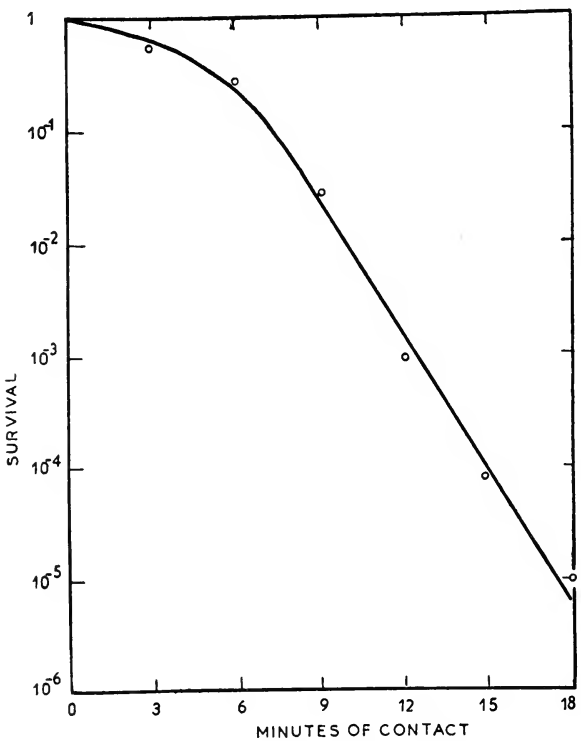


FIG. 1. Inactivation of growing *Esch. coli* B by succinic peracid.

(in order to eliminate the peroxide) and then plated on nutrient agar for colony counts. All survival curves were of the multiple-hit type (Fig. 1). The results can be summarized as follows:

(1) Sensitivity to peroxide varies as sensitivity to radiation.

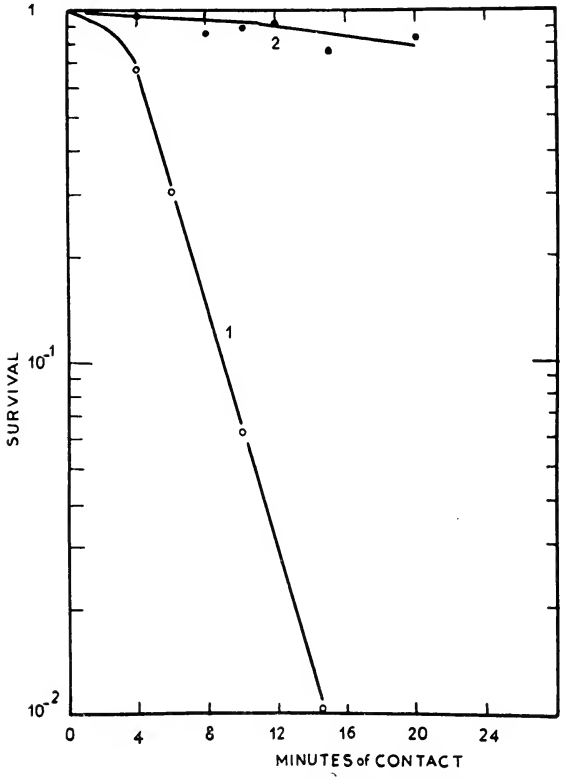


FIG. 2. Inactivation of (1) growing and (2) resting *Esch. coli* K12S by cumene hydroperoxide.

All bacteria are more sensitive in the growing than in the resting stage (Fig. 2). If one compares several strains, one finds not only the same order of increasing sensitivity to peroxide and to radiation (B/r → K12 → B), but even the same

ratios. For example, strains B/r and K12S are equally sensitive both to radiation and to peroxide; B/r is about 6 times as resistant as B to peroxide, as it also is to radiation.

(2) There is no photorestoration after peroxide treatment. On the contrary, Dr. C. C. Brinton observed that under certain conditions doses of light, which are harmless when given alone, may strikingly increase the lethal effect of pretreatment by peroxide. This interesting fact, which will be published, recalls a former observation by Latarjet and Miletic (1953).

(3) The rate of inactivation very much depends on experimental conditions. It rapidly increases with temperature. It decreases when the bacterial concentration increases, soluble organic compounds of the washed suspension and killed cells providing efficient protection to the survivors.

Inactivation of bacteriophage by peroxide

Naked phage is very sensitive to peroxide. Inactivation proceeds at a rate which is either exponential or of low multiplicity of hits (Fig. 3).

(1) Sensitivity of phage may be influenced by its concentration, but this effect may depend on whether or not the phage has been purified. A T2 lysate in 56 synthetic medium containing 5×10^{10} particles per ml. was dialysed against distilled water, then diluted. Each dilution was treated for 15 minutes at 18° by 2×10^{-5} succinic peroxide (Table I).

Table I

Log phage concentration per ml.	T2 *survival per cent*	
	normal lysate	*dialysed lysate*
9	70	
8	12	16
7	0·12	2·4
6	0·22	2·3
5	0·05	1·2
4	0·01	4·7

In the dialysed lysate, sensitivity is independent of concentration below 10^8; but in the normal sample, it steadily increases with dilution, and becomes much greater than in the purified lysate. This fact (and others which we encountered in

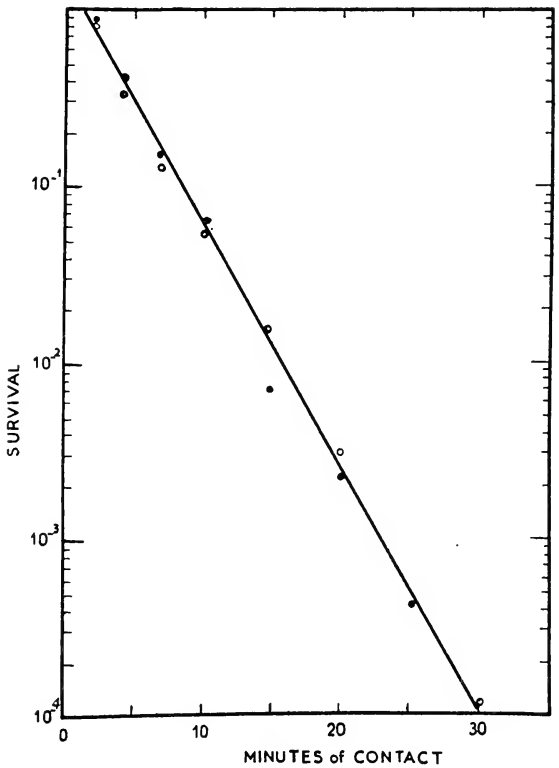


FIG. 3. Inactivation of phage T2 by succinic peracid.

other types of experiments) shows that toxicity of peroxide may increase in the presence of some organic substances. We believe that when oxygen is present these substances can be peroxidized by chain reactions, thus increasing the titre of peroxide groups. This point should be studied, for example by removing oxygen before peroxide treatment. One should

also consider, in the case of T2, the possible inactivation of a phage inhibitor (Sagik, 1954) which would influence the apparent rate of inactivation of the phage itself.

(2) A large phage like T2 can be inactivated by damage to the DNA, or to the tail, or both. If DNA only is damaged, the phage still attaches itself to its bacterial host, and kills it, without multiplying. If some specific site at the tip of the tail is knocked out the phage does not attach, or attaches in such a fashion that it loses simultaneously its infective and bactericidal powers.

It is known that X-rays attack both sites at different rates depending on whether the radiation acts through direct or indirect effect. When it acts indirectly both sites have about the same sensitivity, but when the direct effect becomes more marked the relative resistance of the tail increases much more than that of the DNA. For example, in T6 inactivated by pure direct effect, the DNA is 28 times as sensitive as the bactericidal activity (Latarjet and Frédéricq, 1955).

By treating a concentrated suspension of T2 in buffer (10^{10} particles per ml.) with succinic peroxide, Dr. Maxwell found that both sites are attacked, and that the DNA is about 2·5 times as sensitive as the other site. This ratio is about the same as that found by Watson (1950) on T2 irradiated by X-rays in 0·8 per cent broth. It should be of interest to examine this aspect of peroxide-treated phage. Arber and Kellenberger (1955) have already observed some morphological changes in T2 treated with hydrogen peroxide.

(3) The sensitivities of different phages have been compared in numerous experiments. The temperate phage λ, which has the same size as the virulent phage T2, but a smaller content of DNA, is much more resistant to peroxide than the latter, as it is more resistant to radiation. Among the virulent phages of the T-series, T5 is the more resistant; then come the three T-even phages with about the same sensitivity. But, contrary to what happens with radiation, the smaller phages T1, T3, T7 are more sensitive, possibly because of the lack of a thick membrane around their DNA; for example, 10^{-4}

cumene peroxide after 15 minutes contact at 37° left 18 per cent of λ and 0·4 per cent of T2; 2×10^{-5} succinic peroxide after 5 minutes contact at 18° left 24 per cent of lyophilized T4, and 0·2 per cent of lyophilized T1.

Inactivation of bacteria-bacteriophage complexes by peroxide

Bacteria-bacteriophage complexes are very sensitive to peroxide, especially during the first half of the latent period. As soon as new intracellular mature phage is formed, the infective power of the complex becomes more resistant. In the following experiment, B-T2 monocomplexes were brought into contact, at various times during the latent period, with 10^{-4} cumene peroxide at 37° for 10 minutes, then diluted and plated for survival (Table II).

Table II

Time at beginning of contact (minutes after infection)	*Survival per cent*
3	0·21
6	0·18
9	0·20
12	0·16
15	27

This situation is similar to that observed in complexes treated with strongly illuminated nutrient broth (Latarjet and Miletic, 1953). In this last instance, it had been found that complexes which survive either u.v.-irradiation or contact with illuminated broth, are damaged in such a way that their latent period is lengthened, and their yield in new phage particles lowered. The same partial damage has been observed by Dr. C. C. Brinton in B-T2 complexes treated with sub-lethal doses of cumene peroxide.

The behaviour of the peroxide is so similar here to that of radiation, that an even more specific similarity has been looked for. Phage λ and its indicator bacterial strain K12S are

respectively more radioresistant than phage T2 and its host B. However, the K12S-λ complex is as radiosensitive as the B-T2 complex. This is due to the fact that the "capacity" of K12S to grow λ is far more sensitive than the capacity of B to grow T2. As a matter of fact, in both X- and u.v.-irradiation, if one adds the cross-sections of λ and of the capacity of K12S, one finds roughly the cross-section of T2, the cross-section of the capacity of B being negligible (Latarjet, unpublished).

A situation similar in all respects has been found with cumene peroxide: K12S and λ are respectively more resistant to peroxide than B and T2. However, the infectivities of K12S-λ and B-T2 complexes have about the same sensitivities.

Such parallelism, concerning a very specific biological situation, appears to be very significant for the radiomimetic character of peroxide.

Posteffect after treatment with peroxide

A posteffect has been observed by Alper (1954) after X-ray treatment of bacteriophage. In my laboratory, Miletic (1955) found that under strong illumination, nutrient broth becomes toxic for B-T2 complexes, and that this toxicity proceeds for a certain time after the treated complexes have been washed and resuspended in an inert medium. Catalase suppresses this posteffect. A similar phenomenon has been obtained after peroxide treatment of B-T2 complexes. When the treated complexes are diluted and plated on a minimal synthetic medium, the toxic effect initiated by the peroxide proceeds in such a way that the same final number of survivors is obtained independently of the time of plating. This number is the result of the immediate effect plus the posteffect. On the other hand, when the complexes are plated on broth agar supplemented with catalase, the toxic effect is stopped immediately; the final number of survivors shows the actual figure at the time of plating.

The following experiment, carried out in my laboratory by

Dr. C. C. Brinton, illustrates this finding: 7 minutes after infection, B-T2 monocomplexes growing at 37° C in synthetic 56 medium, are mixed with 10^{-4} cumene peroxide. After 5 minutes of contact, the suspension is diluted 1000-fold in buffer. From time to time 0·1 cc. is spread:

(a) on broth agar supplemented with catalase, in the presence of an excess of bacteria grown in broth;
(b) on 56 agar in the presence of the minimum number of bacteria required to give plaques.

After incubation, the number of plaques gives the number of surviving complexes plus a few free T2. The number of the latter is determined in parallel controls which have been plated after elimination of the complexes by chloroform treatment (Table III).

Table III

Time elapsed after dilution of peroxide (minutes)	*Number of surviving complexes*	
	(a) on broth agar + catalase	*(b) on 56 agar*
4	165	10
9	102	4
22	53	0
30	26	0
40	13	0
55	2	0
100	0	0

One sees that the number of survivors in the (b) series is almost constant and very soon reaches the minimum which, in the (a) series, is obtained only after 100 minutes, during which a constant posteffect shows up.

It appears likely that this posteffect is similar to that observed after radiation treatment. This conclusion reinforces the idea that the radiation posteffect is mediated by radio-formed organic peroxides. However, the possibility is not

ruled out that peroxide immediately absorbed by the bacteria could slowly diffuse through the bacterial body towards the sensitive sites of the complex, a process which could also account for the observed effect.

Part-inactivation of bacteriophage

Indications that ionizing radiations and some chemicals can increase the sensitivity of biological systems to further toxic

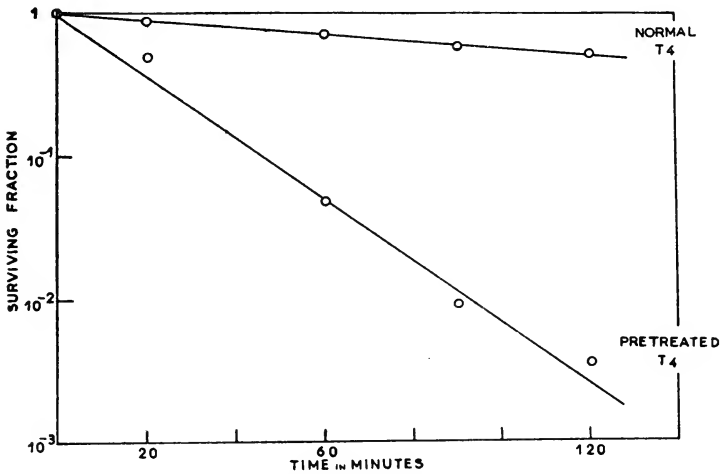


FIG. 4. Inactivation of normal and cumene-hydroperoxide pretreated phage T4 by ascorbic acid.

effects have now been obtained by several workers. In particular, Alper (1955) has shown that bacteriophage which has been irradiated in dilute suspension is more sensitive to inactivation by reducing agents. It seemed of interest to investigate to what extent organic peroxides could replace ionizing radiation in the production of part-inactivated phage. This investigation is being carried out in my laboratory by Dr. D. Maxwell who has already observed the following results.

The work of Alper was done on phages S-13 and T3. Dr.

Maxwell found that X-irradiation also part-inactivates the larger T-even phages of the T series. Working with purified T4, he has studied the inactivation by ascorbic acid of phage pretreated by peroxides.

Succinic peroxide, although inactivating the phage, failed to produce part-inactivated particles. In contrast, however, cumene peroxide was found to produce part-inactivated phage. Concentrated phage T4 was treated for 5 minutes with 10^{-3} cumene peroxide, which produces about 50 per cent inactivation. The phage was then diluted to stop further action of the peroxide, and then treated with 10^{-5} M-ascorbic acid. Fig. 4 shows that the rate of inactivation of the pretreated phage is greater than that of normal T4.

It is of interest that cumene peroxide produces (while succinic peroxide does not) a very typical change; ionizing radiation also produces this change when acting under conditions of indirect effect. Experiments currently being carried out by Dr. Maxwell seem to indicate that most of the part-inactivation is due to a change occurring in the DNA, although there may be a slight effect on the tail.

Mutagenicity of succinic peroxide

Mutagenic effects by some organic peroxides have been observed in *Neurospora* by Dickey, Cleland and Lotz (1949). Moreover, mutagenicity of some irradiated organic media can be attributed to long-lived radioformed organic peroxides (Wyss *et al.*, 1950). Preliminary experiments carried out with succinic peroxide indicate that such mutagenic activity cannot be observed with all peroxides nor necessarily in all biological systems.

(a) Dr. R. F. Kimball (1955, personal communication), of Oak Ridge, exposed *Paramecia* to peroxide for 10 minutes at 26° C in the dark. Following exposure, individual *Paramecia* were isolated and several days later, groups of 25 autogamous animals were isolated from each treated animal. Those exautogamous clones which reached a maximum population

in 4 days were considered normal. The percentage of normal clones was inversely related to the number of mutations.

The peroxide proved to be very toxic. Very few animals survived exposure to 12 μg./ml., but almost all survived 10 μg./ml. Altogether, Dr. Kimball had 25 autogamous clones from each of 344 treated animals of which 224 were exposed to 10 μg./ml. and 40 each to 12, 8, and 6 μg./ml. The percentage of normal exautogamous descendants from these 344 treated animals was 96·0, against 97·5 in the controls. The difference is not significant.

(b) Dr. Luzzati and M. R. Chevallier (1956, personal communication), of Strasbourg, used resting *Esch. coli* strain B. Full-grown bacteria in broth were washed and resuspended in buffer in the presence of 1×10^{-5} to 2×10^{-4} peroxide. Contact was maintained during 30 minutes at 37°. The survival ranged from 0·3 to 10^{-6}. After contact, the bacteria were washed and plated (1) for B/1 mutants, resistant to phage T1 (end-point mutations); (2) for B/Sr mutants, resistant to streptomycin. No induced B/1 mutant was ever observed, whereas the treatment induced up to 4000 B/Sr mutants out of 10^8 survivors.

This is one more example of mutagenic specificity (Demerec and Cahn, 1953).

Inactivation of pepsin by peroxide

Most biological DNA is usually combined with protein. We have seen that the hereditary material of bacteriophage is very sensitive to peroxide, and it will be seen (p. 289) that pure DNA of bacterial origin is even more sensitive. In order to get some idea of what might happen to the protein moiety of nucleoprotein treated with peroxide, a first series of tests has been carried out on pepsin in this laboratory by Dr. Monier.

Crystalline Armour pepsin (P.M. 35,000) was dissolved, at a concentration of 5×10^{-6} M in 0·1 M-acetate buffer. Succinic peroxide was added, and after a certain time of contact was removed by dilution. Proteolytic activity on casein was

determined by Anson's method (with a precision of 3 per cent) before and after treatment. Table IV groups the main results obtained so far:

Table IV

Time of contact (minutes)	*Concentration of peroxide (molar) pH 5·2*			
	6×10^{-5} *at* 35·5°	6×10^{-4} *at* 35·5°	6×10^{-3} *at* 35·5°	6×10^{-3} *at* 0°
	Proteolytic activity			
0	100	100	100	100
1	117	123	122	
30	125	115	95	122
60	111	101	80	115
120	109	84	54	105
240		80	39	90
360		71	37	
20 hours				88

Inactivation is only slightly increased at pH 3·6. Two main facts may be pointed out:

(a) the very great resistance to peroxide of the enzymatic activity, when compared with that of DNA;
(b) the slight enhancement of enzymatic activity by short exposures.

Inactivation of a transforming agent by peroxide

Transforming agents (or transforming principles, TP) are pure DNA of bacterial origin, endowed with specific biological properties, which can be extracted and transferred without losing their activity. When accepted by a suitable cell, a transforming agent endows this cell with a character possessed by the strain from which it has been extracted. The "transformed" cell perpetuates this character. One unit of the agent can be detected by the formation of one transformed bacterial clone. Therefore, such agents provide a unique material for

quantitative experiments on the actions of radiations or other aggressors upon the specific biological activity of DNA.

In collaboration with Miss N. Cherrier, we have recently undertaken a first series of experiments. Our TP, extracted from a streptomycin-resistant strain of *Pneumococcus* and then purified, showed a titre of 10^6 transforming units per μg. of DNA. A solution containing 6×10^{-2} μg. of DNA per ml. of distilled water was brought into contact with the peroxide at various times at 37°. After contact, aliquots were diluted a hundred-fold in the transforming medium in the presence of sensitive bacteria. After the transformations were effected, the bacteria were plated in triplicate on agar supplemented with streptomycin. The transformed clones were enumerated after 2 days of incubation. Controls were done in order to ascertain that the hundred-fold diluted peroxide remaining in the transforming medium did not curb the transformations.

The following results have been obtained so far.

(1) Surprisingly, cumene peroxide at concentrations ranging from 10^{-5} to saturation (about 10^{-3}) produced no noticeable inactivation after contacts of from 1 to 3 hours, either in the dark, or in visible light.

(2) However, succinic peroxide proved to be extremely toxic:

(a) control experiments showed that, at a concentration of 5×10^{-6} in the transforming medium, the peroxide decreased the number of the transformations induced by untreated TP to about 20 per cent of the normal value. At a concentration of 1×10^{-7}, there was no further toxicity. On the contrary, the peroxide increased the number of transformations by a factor of about 1·5; this recalls the previously mentioned increase in the activity of pepsin (see p. 288);

(b) contact with TP in distilled water was done at peroxide concentrations of from 3×10^{-8} to 1×10^{-6}. In all experiments, there was a very sharp exponential inactivation during the first few minutes of contact,

leaving 10 per cent or less of active units, the inactivation of which proceeded exponentially also, but at a much lower rate (Fig. 5). This remarkable feature has been constantly observed in the inactivation of the same TP either by u.v. (Latarjet and Cherrier, unpublished) or by X-rays. The parallelism between X-radiation and

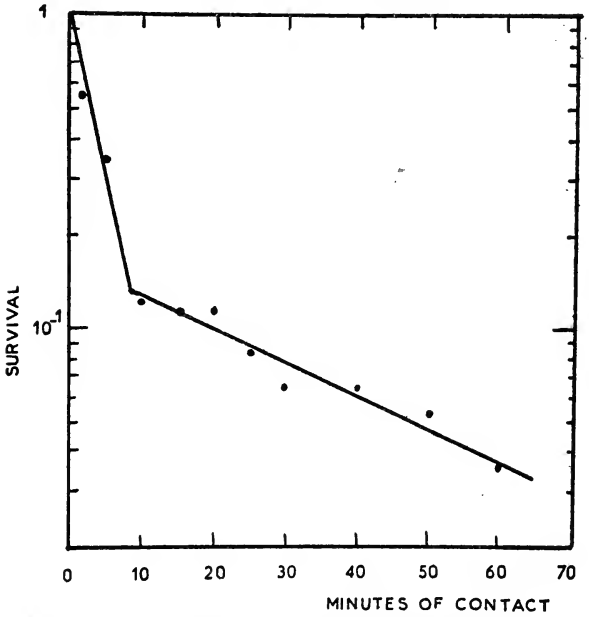


FIG. 5. Inactivation of TP/sr by succinic peracid.

peroxide is, here again, so striking that the results obtained with the former acting by direct effect on the same material should now be mentioned, with special emphasis on the radiobiological aspect of this work.

Inactivation of a transforming agent by the direct effect of X-radiation

This work has already disclosed a number of facts which have been or will be published in detail (Ephrussi-Taylor and

Latarjet, 1955, 1956). Attention will be called here briefly to four points.

(1) *Inactivation.* Non-aggregated DNA ("normal" TP), obtained as the supernatant of a purified and centrifuged preparation, when irradiated in frozen 10 per cent yeast extract (direct effect), yields a typical broken inactivation

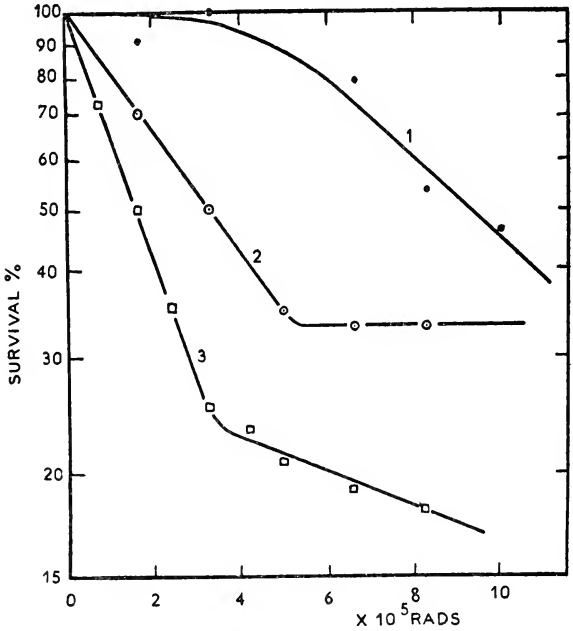


FIG. 6. Direct X-ray inactivation of TP/sr.
1: Aggregated TP, preparation B1; 2: both preparations treated with urea, and normal non-aggregated TP; 3: aggregated TP, preparation B2.

curve (Fig. 6, curve 2). The majority of the units are inactivated at a rate which corresponds to a target with a molecular weight of about 5×10^5. This is less than one-tenth the weight of the whole DNA fibre, as measured by Dr. Doty on the same preparation by light diffusion. The other units display such a high resistance that the corresponding target would not include more than a few hundred nucleotides.

This dualism is not due to genetic heterogeneity, since DNA extracted from bacteria transformed by the resistant units gives the same broken curve. It is not due to aggregation, as will be seen below, since urea, which breaks the aggregates, does not change this curve.

Dr. Ephrussi-Taylor suggests that an explanation might be looked for in two different mechanisms for inactivation. The sensitive one would damage some structure of the molecule-fibre which is needed in one step of the transformation process, such as a successful integration in the bacterium. The resistant one would damage the fundamental structure actually endowed with the biological specificity. This structure should thus be exceedingly small.

(2) *Aggregation and energy transfer.* We used two samples prepared by Dr. Simmons, in which DNA was aggregated. The first one (B1) gave an inactivation curve (Fig. 6, curve 1) with a long plateau and a slope similar to the initial one of "normal" TP (curve 2). We can consider in this case that each aggregate, although containing a number (10–20) of units, acts as one transforming particle when brought into contact with a sensitive bacterium. The type of aggregation is such that each unit is individually inactivated by radiation, i.e., that energy transfer, if it exists, is insufficient to spread throughout the aggregate. When this preparation was treated with 5 M urea, its titre rose, its viscosity increased, and its inactivation curve became similar to curve 2 of normal TP.

The second sample (B2) was actually the centrifugation residue of the preparation, the supernatant of which had formerly been used as normal TP. It gave the inactivation curve 3 of Fig. 6, similar in shape to that of normal TP, but with a two- to threefold increased sensitivity. When treated with urea, this sample too gave the normal curve 2. We may consider that there exists a second type or degree of aggregation, of lower multiplicity than the first one (2–3 normal units), which is also dissociated by urea, but within which radiant energy migrates in such a fashion that the whole cluster may be inactivated by a single hit occurring within

any one of its units. Although the concept of energy transfer within a single fibre is familiar, that of transfer between fibres, the links of which are still unknown, carries more subtle implications. It recalls the transfer between enzyme and substrate postulated by Setlow (1955) in order to explain the increased radiosensitivity of hyaluronidase when combined with hyaluronic acid.

However, it remains possible that, in such a cluster, a hit results in the formation of a very stable cross-link between the aggregated fibres, and that this cross-link prevents successful incorporation by the asssay bacterium.

(3) *Protection against direct and indirect effects.* It has been suggested recently (Alexander and Charlesby, 1954) that radiant energy could be transferred from an absorbing macromolecule to surrounding solutes. This process would bring some protection against the direct effect of radiation upon the macromolecule. We have observed that:

(a) In the liquid state, TP is much more resistant in 10 per cent than in 0·1 per cent yeast extract.
(b) It is still more resistant in frozen 10 per cent and 0·1 per cent yeast extract as well.

We are inclined to interpret these results as follows:

(a) In the liquid state, increasing the concentration of yeast extract gives additional protection of TP against the indirect effects of the radicals produced in water. The form of the DNA fibre, with a great surface: volume ratio, explains the high sensitivity to the indirect effect.
(b) In the solid state, there is no noticeable energy transfer from DNA to the components of the extract. In the present case, there is no protection against the direct effect, as far as physical processes which take place in the solid state are concerned.
(c) Freezing provides additional protection by preventing migration of active distant radicals from water to DNA, without preventing them from recombining before the system is melted.

(4) *Influence of dissolved oxygen and hydrogen.* We have observed that the X-ray sensitivity of TP in liquid 1 per cent yeast extract, a condition where the indirect effect is predominant, is influenced neither by oxygen nor by hydrogen. The rate of inactivation is the same (a) under normal conditions (saturation with oxygen); (b) after oxygen has been removed; and (c) after new saturation with hydrogen. Apparent lack of effect of hydrogen may be due to the fact that the high doses used can themselves produce a lot of hydrogen in the solution. We are dealing here with the specific biological activity of a DNA molecule. Its inactivation may result from some kind of structural change of a nature resembling those involved in gene radiomutations, which, as a matter of fact, are also oxygen-independent. It follows that radiobiological effects sensitive to the presence of these gases should not be considered as the end-results of primary injury to DNA. In particular, those radiation-induced mutations which display high oxygen dependence (chromosome breaks), are likely to result from primary attack upon other material than DNA.

Summary and conclusions

Cumene hydroperoxide and succinic peracid have been used on bacteria-bacteriophage, and on a transforming agent of *Pneumococcus* (TP). The effects observed have been compared to those produced by radiation under similar conditions.

1. Both peroxides inactivate bacteria. Sensitivities of different bacteria to peroxide are of the same order as their sensitivities to radiation.

2. Bacteriophages are inactivated by peroxides. After contact, damage is observed both on the ability of the phage to kill its host, and on its ability to multiply after attachment to the host has taken place.

3. A posteffect similar to that produced by X-radiation has been observed in bacteria-bacteriophage complexes treated with organic peroxide.

4. Phage T4 treated with a sublethal dose of cumene

peroxide is sensitized to the toxic effect of a reducing agent such as ascorbic acid. This "part-inactivation" is similar to that produced by X-radiation. Succinic peracid does not part-inactive T4.

5. Some mutagenicity has been observed for succinic peracid in *Esch. coli* B, but not all mutations are produced.

6. Pepsin is slowly inactivated by peroxide after short exposures have slightly enhanced its enzymatic activity.

7. A TP (DNA) which endows *Pneumococcus* with resistance to streptomycin, has displayed an extremely high sensitivity to succinic peracid, but remained undamaged after treatment with cumene hydroperoxide. The inactivation curve is broken, as it is in the case of X-ray inactivation, the break being due neither to aggregation of DNA fibres, nor to genetic heterogeneity. It is postulated that inactivation results from two different mechanisms. A quantitative analysis of the X-ray curve leads to the hypothesis that the particular structure within the DNA fibre actually endowed with the transforming activity is exceedingly small.

8. X-ray inactivation of several preparations of TP has disclosed two types of aggregation of DNA fibres, which is disrupted by urea.

9. There is no oxygen effect in the X-ray inactivation of TP, and no protection against the direct effect of radiation by organic solutes.

10. These results, of preliminary character, stress the radiomimetic activity of organic peroxides and, therefore, their possible rôle as intermediates in some actions of radiation in living material. They also disclose that the behaviour of a peroxide and of a peracid sometimes displays striking differences.

Acknowledgements

I hope that the preceding text has clearly underlined the paramount part played by my collaborators in the present work. It is a great pleasure for me to thank them here:

Dr. B. Ekert, who performed the peroxide titrations, and, with Dr. R. Royer, synthesized the succinic peroxide; Drs. D. Maxwell, C. C.

Brinton and R. Monier, who obtained some of the most significant results on bacteriophage, bacteria and pepsin; Dr. H. Ephrussi-Taylor and Miss N. Cherrier, who participated in the experiments on the transforming agent; Mr. P. Morenne and Miss G. Hiernaux for their excellent technical assistance; Dr. J. Jagger and Mrs. P. Monnot for their help in preparing the English text.

REFERENCES

ALEXANDER, P., and CHARLESBY, A. (1954). *Nature, Lond.*, **173**, 578.
ALPER, T. (1954). *Brit. J. Radiol.*, **27**, 50.
ALPER, T. (1955). *Radiation Res.*, **2**, 119.
ARBER, W., and KELLENBERGER, E. (1955). *Schweiz. Z. Path.*, **18**, 1118.
DEMEREC, M., and CAHN, E. (1953). *J. Bact.*, **65**, 27.
DICKEY, F. H., CLELAND, G. H., and LOTZ, C. (1949). *Proc. nat. Acad. Sci., Wash.*, **35**, 581.
DUBOULOZ, P., MONGE-HEDDE, M. F., and FONDARAI, J. (1947). *Bull. Soc. chim. Fr.*, **800**, 900.
EPHRUSSI-TAYLOR, H., and LATARJET, R. (1955). *Biochim. biophys. acta*, **16**, 183.
EPHRUSSI-TAYLOR, H., and LATARJET, R. (1956). In press.
LATARJET, R., and FRÉDÉRICQ, P. (1955). Virology, **1**, 100.
LATARJET, R., and MILETIC, B. (1953). *Ann. Inst. Pasteur*, **84**, 205.
MILETIC, B. (1955). Thèse Université, Paris.
SAGIK, B. P. (1954). *J. Bact.*, **68**, 430.
SETLOW, R. (1955). *Ann. N.Y. Acad. Sci.*, **59**, 471.
WATSON, J. D. (1950). *J. Bact.*, **60**, 697.
WYSS, O., HAAS, F., CLARK, J. B., and STONE, W. S. (1950). *J. cell. comp. Physiol.*, **35**, Suppl. 1, 133.

DISCUSSION

Butler: Do Stent's experiments, in which he produced a great many disruptions of nucleotide threads without losing the activity, fit in well with yours?

Latarjet: Yes.

Spiegelman: It is remarkable that this estimation of the order of magnitude of a tenth fits in with two quite independent experiments. One is Benzer's whose gene size is down to 10 nucleotide pairs, and the other is Stent's experiment where with ^{32}P decay about one out of ten leads to a lethal event.

Gray: Another way of putting what has been said is that in the target the volume is not necessarily aggregated. A second point is that the first part of the curve seems to me to indicate that one in ten of the particles are not subject to this inhibition of penetration into the bacillus. In some way they must be different from the other nine, since they are not appreciably inactivated by the doses which you used.

Latarjet: They behave differently after irradiation.

Spiegelman: So you really have a heterogeneous population with respect to the sensitivity to attachment.

Latarjet: I wouldn't say attachment.

Spiegelman: That's just an analogy.

Alper: Whether or not radical reactions in the classical sense could take part in events inside the cell, it seems that there is something rather mysterious going on in the radiation chemistry of various *in vitro* systems. Dr. Dale has told us that all the enzymes that he has investigated in dilute solution showed no oxygen effect. This seems all right if the changes are due to — OH radicals, because the presence of oxygen does not affect the number of these radicals. Whether any of these systems have also been examined in the presence of hydrogen, I don't know; but certainly it does seem rather mysterious in Dr. Latarjet's case because he has tried oxygen and he has also tried hydrogen. Now if you are going to regard the agent responsible as the — OH radical (and I should think on any sort of radiation chemical picture we must still regard indirect action in *in vitro* systems as due to radicals), it is very difficult to see how you can get indirect action which is not affected by the hydrogen, which converts — OH radicals into hydrogen radicals.

Latarjet: May I say that perhaps we have an artifact in the experiment carried out in the presence and in the absence of hydrogen, since the material was very radiation-resistant. We worked with doses of up to 500,000 r and, in the absence of gas, we did not prevent the formation of a great amount of hydrogen radicals in the medium due to radiation. Therefore, there was perhaps in this instance no experiment at all in the absence of hydrogen; all were probably in the presence of hydrogen. That might account for the fact that we did not find any difference with and without hydrogen.

Alper: This means that your *in vitro* indirect effect could be due to hydrogen radicals, and this is the only thing it could be that would fit with your experiments.

Alexander: I don't think that interpretation is necessarily valid, if we accept some of Dainton's latest work that the reaction between hydrogen and — OH radicals to give H atoms is not very favourable. If there is nothing else for the — OH to react with then this reaction will occur, but in Latarjet's system where there are great quantities of proteins and other organic matter, it is most unlikely that the — OH radicals will interact with dissolved hydrogen. We found that as little as 0·02 per cent of organic matter prevented the reaction of hydrogen at atmospheric pressure with H atoms (Alexander, P., and Fox, M. (1954), *Trans. Faraday Soc.*, **50**, 605).

Latarjet: Our preparation was highly active; we had one million transforming units per μg. of DNA. That is a great advantage and therefore we worked with an amount of DNA whch was about 10^{-2} μg. per g.

Spiegelman: But it was protected.

Latarjet: It was very much protected by 10 per cent yeast extract.

Alper: This means that the hydrogen cannot react with the — OH because there is a lot of organic material present to grab the — OH. But

then there are still enough — OH radicals to react with the active material, and presumably the hydrogen concentration is quite a lot greater still than the number of molecules of protected material.

Alexander: The two peroxides studied by Latarjet are chemically very different, and a great deal is known about their reaction with proteins. The organic peracid is the type of substance which has enabled Sanger to do his insulin work, i.e. the type of material which reacts very rapidly and very specifically with SH and with –S–S– groups to convert them to sulphonic acid. Cumene peroxide is quite different: it also reacts with protein and also attacks the SH and –S–S– groups but it does not give sulphonic acid in quantitative yield, it gives largely sulphate. Unlike the peracid it attacks the peptide bond. Chemically these two substances show such different behaviour that one would expect their biological effects to be quite different.

Latarjet: It is a constant phenomenon.

Dale: Dr. Latarjet, in your first slide (on pretreatment), how did you protect yourself against the aftereffect? When you treated your material and then exposed it to ascorbic acid or *vice versa*, couldn't there have been an aftereffect, or what precautions could you have taken? The time was roughly the same as on your aftereffects slide.

Latarjet: It was such a slight dose (we had 50 per cent survival) that the aftereffect was not great, and it was the same in both the control and the treated samples.

Popjak: Dr. Latarjet, with regard to the radiomimetic effect of these peroxides in mice, you said that you gave the substance in oil?

Latarjet: We first injected cumene hydroperoxide dissolved in oil. Then we had some trouble because the toxicity of the solution increased with time. It seemed that cumene peroxide initiated some peroxidation in the oil itself. Therefore, we turned to the water-soluble persuccinic acid. We are now coming back to cumene, using a new organic solvent which has been synthesized in our laboratory.

Popjak: I wonder whether the effect might be due to the peroxidating action on the highly unsaturated fatty acids, and that in effect you might be producing the essential fatty acid deficiency in view of the skin lesions. The effect might be of that type.

GENERAL DISCUSSION

de Hevesy: I feel sure that you all share my view that we experienced a most profitable and exceedingly pleasant meeting. Our thanks are due to all who addressed us and participated in the discussion, but first of all to our Chairman, Prof. Haddow, and to the organizer of this meeting, Dr. Wolstenholme, to the assistant secretary, Miss Bland, and to all members of the able and friendly staff of the Director.

If I am permitted to add a personal remark, I wish to say that I never experienced a more pleasant meeting.

We have traversed various territories and it is difficult to decide which of the countries passed has the most beautiful scenery. One may say that nothing was more fascinating than following the path and fate of seeded marrow-cells as was done by Dr. Loutit which revealed among others the powerful effect of radiation on immunity. It really sounded like a fairytale. Some, however, may give preference to the discussion of the great variety of changes in the enzymatic pattern produced by irradiation, in which various speakers participated, and to the presentation of the philosophy of such happenings put forward in such a fascinating way by Prof. Krebs. Protection was one of the main fields of discussion. Formerly, a geneticist as far as he was interested in the application of X-rays was anxious to produce the maximum number of mutations. By working on these lines very important results were obtained, among others in the field of agriculture. Quantity and quality of crops were improved. I doubt if any geneticist envisaged in those days that the time might come when the main concern will not be to produce mutations by irradiation but protect against them. Dr. Hollaender reported results of his and his colleagues' endeavour to achieve protection against mutative effects of radiation. This was followed by an animated discussion on different aspects of genetic happenings.

That the basic problem of radiobiology, the site of primary biochemical lesion, is yet unsolved was emphasized by different speakers. Now in view of the great variety of enzymatic changes produced by irradiation one may be inclined to consider such inactivation to be the primary radiation damage. What makes one doubt the correctness of this assumption is the fact that while the same enzyme when present in a radiosensitive organ can easily be inactivated, when located in a less radiosensitive one proves to be refractory to even large doses. We were told by Dr. Van Bekkum that when the rat is

irradiated with 100 r the oxidative and phosphorylating power of its mitochondria gets markedly reduced and that 50 r suffice to obtain similar effects in the thymus. But if we expose rats to much larger doses the mitochondria extracted from their liver go on to oxidize and phosphorylate at a normal rate. Now you can of course say that the composition of the liver differs from that of the spleen and thymus; that the liver contains constituents having a powerful protecting effect which prevent radiation energy reaching the sensitive spots. You may also say that the enzymatic pattern differs in the liver from that of the spleen or thymus, recalling the considerations put forward by Prof. Krebs and that conditions for enzyme inactivation are more favourable in the last-mentioned organs. But the above-mentioned difference is not only shown when comparing liver with spleen or thymus but also other moderately sensitive organs with very sensitive ones. Thus we have to consider the possibility that inactivation of enzymes is preceded by cell lesion. As to the latter, a conspicuous parallelism is shown between radiation sensitivity and rate of DNA formation in the organ considered. You can easily find exceptions to this regularity. Lymphocytes in which no DNA formation takes place are an example, some plant seeds, some plants. In view of the very great number of parameters involved it is very difficult to find any regularity which is valid without exception. It has been known for many years that cells exposed to irradiation often die when trying to divide. Li emphasized this point in his classical book. Our knowledge of this type of cell death was enlarged by investigations reported by Forssberg in his address. He has shown that by irradiating mice with ascites tumour the formation of DNA in the tumour cells is blocked, at the same time synthesis of several metabolites goes on. Such a selective influence is bound to have serious consequences. DNA formation being obstructed, the cell cannot divide and thus it has to accommodate all additionally formed metabolites in the mother cell which correspondingly swells. At a later stage, when the power of the cell to synthesize DNA recovers, the cell can divide. But division of such an abnormal cell is often fatal.

When considering the above-mentioned exceptions we must take into account the fact that exposed cells may die unconnected with division processes. Even unexposed erythrocytes die and their death may be accelerated by interference with oxygen supply, production of haemolysing substances and other agencies. It was shown by Howard and Pelc that irradiation may interfere with a very late phase of the mitotic cycle in which the full DNA complement of the cell is already reached, and Dr. Howard told us that she considers this type of interference to be the primary one, a view against

which some evidence was brought by Dr. Lajtha. Recent results obtained by Mazia when studying connection between RNA synthesis and cell division suggest that this late radiation effect may possibly be interference with RNA formation.

The mechanism responsible for DNA synthesis is not known but it is quite probable that its formation necessitates the presence of intact DNA protein molecules and that interference with these and possibly also with the RNA protein molecule are the primary cell lesions. In the mammalian organism unique size and length of a DNA protein molecule much favour uptake of radiation energy. We were told by Prof. Mitchell that he is inclined to consider a macromolecular lesion of DNA protein to lead to a healing of carcinoma.

As to the effect of radiation on the DNA protein present in the tissue, it is just ten years since Errera irradiated nucleated red corpuscles and determined the rigidity prior to irradiation and after exposure to a very massive dose of 5000 r or more. He found the rigidity to be reduced. Quite recently, in Dr. Hollaender's Institute, Anderson carried out experiments with diluted homogenates of thymus, determining their viscosity prior to and after exposure. He succeeded in reducing the viscosity after exposure to only 25 r. It may be purely fortuitous or not—it is difficult to tell—that the dose that will interfere with DNA synthesis in the thymus, as determined by Ord and Stocken, is about the same as that which is necessary to depress its viscosity, thus to depolymerize thymus DNA. Now I just wonder if it would be possible or profitable to carry out with other tissues, experiments similar to those which Anderson did with thymus. Thymus, of course, has the high DNA content which makes it easier to work with, but it may be possible to take other tissues and to investigate if there is any parallelism between the ease with which deploymerization takes place and DNA formation, and thus also between radiosensitivity. We come here very near to a suggestion made by Prof. Mitchell. He remarked that it is possible that the difference in behaviour of radiosensitive and refractory tumours is due to the fact that the refractory tumours contain DNA of low grade of polymerization; correspondingly, irradiation cannot easily produce further changes in these. But even if these conclusions were not to be substantiated, it could be quite possible that depolymerization of the refractory tumour tissue would need a larger dose than that of a sensitive tumour.

It is thus quite possible that enzymic processes are preceded by cell lesion produced by interference with nucleoproteins. But even if this interference with DNA protein and possibly also with RNA protein should prove to be a very important early step, introducing cell damage, it is not necessarily the first one.

Dr. Hollaender mentioned the very rapid irradiation effects produced by change in salt concentration of the surroundings. Perhaps Dr. Hollaender is willing to give us some more details of how this exciting experiment was carried out, and also to tell us if it is possible and advisable to carry out experiments similar to those which Anderson did in his laboratory with thymus tissue, with other types of tissue, and if he expects to find marked differences.

Hollaender: Dr. Gaulden, in previous studies, had demonstrated that if she subjects the neuroblast to hypertonic culture medium, the chromatin of cells in middle prophase takes the appearance of late prophase and the chromatin of late prophase assumes the appearance of very late prophase. These changes occurred in 15 to 30 seconds after the cells were placed in a medium hypertonic to them. This apparent advancement of stages of mitosis was shown to be accompanied by an accelerated mitotic rate.

She then decided to set up some experiments to determine whether the "reversing" action of X-rays on middle and late prophase primarily responsible for mitotic inhibition at low doses could be prevented by subjecting the neuroblast to a hypertonic medium which advances these stages. The results indicated that this can be easily accomplished. Whereas the mitotic rate of irradiated (3 r of X-rays) neuroblast in isotonic medium was depressed down to about 25 per cent of normal level, the mitotic rate of irradiated neuroblast in hypertonic medium was depressed only down to about 75 per cent of normal level. Thus, subjection to hypertonic medium immediately following irradiation resulted in less radiation damage than if isotonic solution is used. It was also found that if the cells were put in a hypertonic medium more than a minute after irradiation had been stopped, they responded to the irradiation in the same manner as irradiated cells in isotonic solution. The most successful tests were accomplished by irradiating these cells in a very slightly hypertonic salt solution and then, immediately after irradiation, putting them in a more definitely hypertonic medium. Under these conditions, one could eliminate practically all the effect of mitotic inhibition produced by X-rays. It is not possible at this time to tell whether the influence of hypertonic medium is due to removal of water from the cell or to increased concentrations of certain inorganic salts or to both. (See the forthcoming paper, "Prevention of X-ray induced mitotic inhibition in grasshopper neuroblasts by post-irradiation subjection to hypertonic culture medium", which Dr. Gaulden is now preparing.)

I believe such an approach with hypertonic salt solution could be used in connection with other radiation work. The important thing, probably, is to get it into the cell very quickly after irradiation has stopped so that the damage is still "reversible".

Now going back to Dr. Anderson's work, which is being extended by Mr. Fisher, of course the original work at Oak Ridge was initiated on the effects on the nucleic acids on the basis of the experiments we had done many years ago in co-operation with Drs. Greenstein and Taylor at the National Cancer Institute and National Institute of Health, when we found that 10–20,000 or 50,000 röntgen were necessary to depolymerize sodium thymonucleate, and it might interest you that the background for the work on the nucleic acids at Oak Ridge was this finding. But it was quickly found out by Dr. Carter and later on by Dr. Cohn that the materials we were working with were either very impure compounds or isolations of mixed materials with which experiments could not be repeated from time to time, and it was finally decided at that time to go more thoroughly into the whole problem of nucleic acids, first of isolation and then structure of the nucleic acids, and we are just starting again to study the effects of radiation on these mixtures or compounds if we can get them pure enough. I think that there are many possibilities, even with the impure mixtures, to follow what radiation will do in them; concentrating entirely on first getting the purest possible compound may not tell us this story. I feel that even these crude extracts may give us more information than the very pure compounds which we finally will have to study; these crude mixtures may tell us much more of what is happening inside the cell. I think Dr. Anderson picked the easiest tissues to handle, as Prof. de Hevesy pointed out. But he plans to go ahead on other tissues too and see if he gets the same type of result.

de Hevesy: Errera needed 5,000 r, Anderson only 50. Errera worked with nucleated red corpuscles of the hen, in which no DNA turnover takes place. So it is not impossible that the tremendous dose difference, 50–5,000 r, is not only due to Anderson's improved technique but the fact that Anderson picked out a very radiosensitive system while Errera worked with a very radioresistant one.

Alexander: I think this difference between Errera and Anderson is largely one of concentrations. One can add as much water as one likes to these nucleoprotein gels and when dilute they show dilution effect typical of indirect action in a very pronounced manner. Before concluding that one system is more sensitive than another we must take concentration into account. Errera's concentration was governed by the physiology of the cell and was quite high. In the test-tube one can handle it at much lower concentrations, and one of the reasons why Anderson could detect such small doses must have been that he worked at concentrations which were much lower than those of Errera.

Butler: With regard to de Hevesy's point about the other tissues,

the rat thymus is rather particular in that the nucleoprotein is not completely dissociated in this salt solution. If you take the same thing with beef thymus you get a much greater degree of dissociation and you don't get this remarkably high specificity.

Haddow: Dr. Howard has put forward a suggestion, namely that Dr. Dale should tell us to what extent he believes his five questions have been answered.

Dale: In my presentation, I deliberately did not express any opinion, giving simply a background survey, and at the end of it I put some questions (p. 33) which involved the two main manifestations of indirect action, the dilution effect and the protection effect, and asked this audience whether there is scope for these two manifestations to explain the possible mode of action of enzymes. One of the main questions, i.e. the fourth question, was answered to a certain extent in the way in which I put it, but Prof. Krebs in his contribution modified this and answered it more or less in this way, that he thought the determining factor is not so much the disturbance of the enzyme itself, but of the substance which is concerned with the enzymatic process, that is of the substrate; and of course the dilution effect will just as well act on that if it acts at all.

I understood from Prof. Kreb's answer to my third question that there is a possibility of a minute amount of available substrates being interfered with at the steady-state, which may be low. The steady-state concentration of the substrates may be a determining factor, and may be responsible for disorganization of consecutive steps in the enzymatic action.

I also asked the question "Are these intermicellar spaces in the inhomogeneous cell structure filled with high concentrations of protective substances?" This is an objection that is usually made, that there are plenty of protective substances which will obviate the indirect action. Now there are model experiments by Stein and one co-author in which he irradiated gelatin gel in which he had incorporated methylene blue, and there was little or no interference with the action of degradation of this methylene blue. In this case one would say, taking the gelatin as the cytoplasmic model for the interior of the cell, that the micellar structure of this gel did not interfere with the indirect action. Indirect action could still take place in the solvent-filled spaces between those gelatin structures. Also, Gordon and his co-worker used agar gels and did not find a protective effect of these on substances distributed in these gels. In answer to my fifth question, I would say quite emphatically that any experiments on these lines are doomed from the start. They cannot give any indication of what is happening, because all that this type of experiment is concerned with is the total amount which is present,

and that is not what matters at all. If anything matters it is the functional part and not the store, and the notoriously small amounts of change by moderate radiation doses in any substance can, of course, have nothing to do with the bulk from which they are changed by radiation.

My first question is similar to that of the separation of substrate and enzyme and—to extend it—the enzyme can diffuse or the enzyme is phase-bound and the substrate is diffusing to it. Evidence has been brought forward by some workers that substrate and enzyme, at least in certain cases, are localized separately and in order to get to each other they have to be in transit of some form.

One more point that I should like to mention is the apparent and, to a certain extent, neglected importance of chain reactions. Some, of course, are known and we have a very interesting example of a dose-rate dependent chain reaction with respect to the liberation of sulphur from thiourea, which at a dose rate of 0·39 has G values of 17,000–20,000 and there is virtually no limit if one goes down still further with the dose-rate. It is also known that for instance, the oxidation of cysteine to the disulphide is a chain reaction, and furthermore (which makes it so difficult to reconcile any scheme devised by physical chemists for any reaction with radicals) that the action of some of the radicals very often must cause changes which lead to new radicals which are probably very difficult or nearly impossible to put into a reaction scheme. That refers to oxidations, to dehydrogenations which are one-step reactions, leaving a radical which again may do something, and the phenomenon I have shown, namely the "changing quotient" (i.e. that the protective power per unit mass of the substance added declines on increasing the concentration) can be explained also by the formation of a radical from protector molecules which again hands on the energy.

Holmes: Dr. Hug and co-worker (Hug, O., and Wolf, I. (1956), Progress in Radiobiology, p. 23. Edinburgh: Oliver & Boyd) made a very useful contribution to the knowledge of the effects of irradiation of systems in a steady state. They irradiated with X-rays luminescent bacteria which were emitting light of a steady intensity. A diminution of light-intensity was shown as soon as the irradiation was begun and the intensity fell continuously as long as the irradiation continued. Directly irradiation ceased the light emission began to recover and became steady at an intensity rather lower than that originally found. The recoverable part of this system was undoubtedly an irradiation-sensitive unit of the light system which was restored by the further activity of the bacteria.

Dale: That is quite possible. There is an American worker too, who works with fireflies. It is a very sensitive reaction.

Popjak: May I contribute to that? I think the real significance of those experiments might be that the phenomenon was observed during irradiation, and Hug has observed the luminescence while the bacteria were irradiated. Immediately on starting irradiation the luminescence decreased, when he stopped irradiation it returned, and so on, although I think that with higher doses the luminescence did not quite return to the original starting value. The importance of this is that it is well worth thinking of experimentation in which we try to look at enzyme reactions while irradiation is going on.

Laser: I have mentioned before that the enzyme notatin is inactivated by X-rays more strongly if irradiation takes place in the presence than in the absence of its specific substrate (glucose). I should add that I conclude that the enzyme is most sensitive to X-ray damage at the stage of a semiquinone.

Dale: Which would fit in with what I mentioned.

Latarjet: Dr. Dale, may I ask you if your chain reaction of thiourea takes place in the absence of oxygen?

Dale: On the contrary, it takes place in the presence of pure oxygen. If you decrease the oxygen tension the dose-rate dependence is not abolished, but the absolute effects are getting smaller and smaller. In the absence of oxygen there is hardly any effect.

Mitchell: One point which we have not discussed at all at this meeting, and which I think might be of interest, is the relative biological efficiency of different radiations for metabolic effects, particularly the comparison of effects of radiation with low and high specific ionization, in biosynthesis of nucleic acids. There are the experiments of K. G. Scott (1946, *Radiology*, **46**, 173) and the more recent experiments of A. Howard and S. R. Pelc (1953, *Heredity*, Suppl. to Vol. **6**, p. 261). I wonder if people consider that further information might be obtained from a special study in this direction.

Howard: To follow up that suggestion with regard to effects of different types of radiation on DNA synthesis in tissues would be extremely useful. We have very little information on this point, and it would give us one obvious means of sorting out the mitotic delay and cell death on the one hand, and the effect on DNA synthesis on the other hand.

Haddow: When Banting discovered insulin, or most probably rediscovered it, about 1922, he was expected by the public to make a great series of further discoveries along the same lines, including a cure for cancer, the study of which he took up shortly thereafter. I remember Sir Henry Dale telling a story of how, round about 1923, he was phoned up in great excitement by a Press reporter to know was it true that Banting had discovered a cure for metabolism. We may not have discovered the cure for metabolism, but we may be on

the way to the control of the disorders of metabolism which are brought about by ionizing radiations.

I think it has been very well worth while coming here for all sorts of reasons, not least to hear Professor de Hevesy say what he said a short while ago. I thought this was a very moving and masterly summary. He was obviously impressed by what he called the fairy tale which Dr. Loutit has told us, and I think we are specially grateful to Dr. Loutit for recalling the early key observations, particularly those of Jacobson, and the later work of Lorenz who is so well remembered in the Ciba Foundation. I have a feeling that the progress of work depends rather on the making of such key biological observations. We must not detract from the skill or prowess of the biochemist; but from my own experience, with all respect to my biochemical confrères, I find that very seldom are they able to direct one to make the discovery. The discovery very often having been made by the biologists, they can then explain it in many cases, or endeavour to do so. And this leads me to another impression I have had during this meeting: that we more and more approach the holistic view of the cell. As Dr. Zamecnik showed so graphically, atoms and molecules tend to mean very little in themselves, and it is the way in which they are put together that really matters. We will all recall for a very long time to come the courage shown by Krebs in throwing his paper and slides out of the window. Lastly I should like, from myself and on your behalf, to tender thanks to Dr. Hollaender, Professor Butler, Dr. Gray and Dr. Wolstenholme, for all their help in the early arrangements of a profitable and memorable Symposium.

AUTHOR INDEX TO PAPERS

SUBJECT INDEX

Printed by Spottiswoode, Ballantyne & Co. Ltd., London and Colchester