THE NITROGEN METABOLISM OF MICRO-ORGANISMS

B. A. FRY

METHUEN'S MONOGRAPHS ON BIOCHEMICAL SUBJECTS

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General Editors: SIR RUDOLPH PETERS, F.R.S.
and F. G. YOUNG, F.R.S.

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PREFACE

LITTLE reflection is required to realize that nitrogen is a constituent of numerous compounds of biological interest, and all acquainted with present-day biochemistry are aware that during the last ten years the former emphasis on the study of the degradation of complex substances has been largely replaced by an active interest in mechanisms of synthesis, and in particular, in the synthesis of proteins and the metabolic role of the nucleic acids. Micro-organisms are proving to be of great value in the unravelling of the routes whereby amino-acids, nucleotides and other compounds are synthesized *in vivo*, and for a long time they have been used with great success in experiments designed to elucidate the functions of the many water-soluble substances now included in the B group of vitamins.

In this monograph an attempt has been made to survey as comprehensively as possible the nitrogen metabolism of micro-organisms and to treat the subject in such a manner as to reflect current trends in modern microbiology. The monograph is based on a series of lectures given in a one-year post-graduate course of microbiology held in the University of Sheffield, and it is hoped that advanced students at other universities and research workers in allied fields will find it a convenient and concise introduction to one important section of microbial biochemistry. If it be thought that some topics receive more attention than they warrant, then the author accepts full responsibility for his choice and defends it on the grounds that these topics either encompass ideas of wider significance or serve to focus attention on how little has really been established. Though the title of the monograph is all-embracing and in the text examples are drawn from experiments with bacteria, fungi, algae and protozoa, the main emphasis is naturally on the first two of these four groups, since most work has been done with species of bacteria and yeasts. There is not space to mention every

organism which has been studied, but the reader's search for additional information should be aided by the books recommended for general reading and the detailed bibliography appended to each chapter.

It is with very great pleasure that I record my thanks to Dr. S. R. Elsden for his interest in the preparation of this monograph and to him and Dr. J. L. Peel for reading the drafts of the various chapters and making many helpful suggestions. I am also grateful to Dr. E. F. Gale, F.R.S., for reading the completed manuscript.

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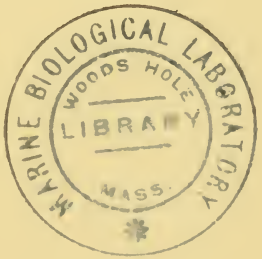

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CHAPTER I

INTRODUCTION

Energetics of biological systems

In recent years the attempts to analyse the energetics of biological systems in terms of established thermodynamic principles have naturally focused much attention on the reactions in such systems which yield energy and those which utilize energy [6]. When energy is supplied to or liberated in a system, there are limitations regarding the conversion of one form of energy into another (Second Law of Thermodynamics). In other words, only part of the energy content of any system is available for doing further work, and this useful energy is termed *free energy*. Chemical reactions in which there is an output of free energy are described as exergonic and those in which there is an uptake of free energy as endergonic. Reproduction, growth and the maintenance of life are all endergonic processes and are therefore intimately associated with mechanisms able to supply them with energy.

It is generally believed that energy becomes available in biological systems as the direct or ultimate result of oxidation reactions [8, 10]. The oxidation of one substance must necessarily be accompanied by the reduction of another and a biological oxido-reduction reaction involves the transfer of hydrogen atoms or electrons [14]. Consequently the substance which is oxidized is sometimes described as being a hydrogen donor [H-donor], whilst the one being reduced is termed the hydrogen acceptor [H-acceptor]. The complete oxidation of any one substance proceeds by one or more simple steps, each catalysed by the appropriate enzyme, and in all known reactions the transfer of hydrogen atoms or electrons to the ultimate H-acceptor is effected by one or more intermediate carriers. In aerobic organisms, molecular oxygen serves as the H-acceptor and, according to the enzyme concerned, the end-product is water or

hydrogen peroxide. Organisms which by chance or by necessity are living in an anaerobic environment must use a substance other than oxygen for this purpose. Such a substance may be derived from the environment (e.g. CO_2, nitrate or acetate) or may be a product of the organism's catabolism (e.g. in the lactic acid bacteria, pyruvate is reduced to lactate).

The esterification of inorganic orthophosphate is an integral part of the mechanism whereby endergonic reactions are able to utilize the energy made available by oxido-reduction reactions. Our conception of this mechanism is mainly due to Lipmann [10], who pointed out that phosphorylated compounds can be divided into two groups according to the amount of energy released by their hydrolysis: some yield about 3,000 cal. per mole whilst others liberate 10,000 to 16,000 cal. per mole. Lipmann proposed that the latter should be known as high-energy (or energy-rich) phosphate compounds and that they contain what he termed high-energy (or energy-rich) phosphate bonds, the hydrolysis of which yields 10,000 or more calories of free energy per mole of inorganic orthophosphate liberated. The significance of certain biological oxido-reduction reactions lies in the fact that they are associated with the formation of energy-rich phosphate bonds: these arise either during the actual oxidation of the organic substrate or else during the transfer of hydrogen (or electrons) to a H-acceptor. In the former case oxidation of the organic substrate is accompanied by its esterification with inorganic orthophosphate and in consequence most of the energy made available by the oxidation reaction is not liberated as heat but is retained in the oxidized substrate in association with the newly incorporated phosphate group. The only known example of an energy-rich phosphate group arising by a non-oxidative reaction is found in *enol*-2-phosphopyruvic acid, a substance formed by the dehydration of 2-phosphoglyceric acid under the influence of enolase. The phosphate groups and their associated energy can be transferred, in the presence of the appropriate enzyme (a phosphokinase), to adenosine diphosphate (ADP), or sometimes to adenosine monophosphate

(AMP), thus forming adenosine triphosphate (ATP) or ADP respectively.

In a biological system, the only known way in which the energy released by an exergonic reaction can be made available to an endergonic reaction, is for the two reactions to be coupled together by means of a substance which participates in both. This is the function of ATP, which by virtue of its high-energy phosphate groups acts as an energy carrier between reactions yielding energy and those utilizing energy. Adenosine triphosphate participates in the latter by reacting with, and thus activating, one of the reactants, and by this means the total free-energy content of the reactants is raised to a value at least approximately equal to, and often far greater than, that of the products. From the standpoint of energy relationships, the conditions are now such as to favour formation of the products, and the utilization of ATP in this manner is accompanied by the appearance of inorganic orthophosphate.

Although it is generally accepted that the energy metabolism of all organisms is associated with energy-rich phosphate bonds, little is known about how they are formed except during the anaerobic catabolism of glucose and pyruvate. The results of contemporary research indicate that co-factors containing thiol groups probably play an important role both in the production of energy-rich phosphate groups and in their utilization, and that the synthesis of thiol esters may be an essential intermediate stage in these reactions (cf. the role of glutathione in the triosephosphate dehydrogenase system [16], and coenzyme A (Co.A) in the synthesis of citric acid and other compounds [2]). A substance having the properties of ATP is believed to be present in all organisms and ATP has in fact been isolated from yeast [cf. 4], green plants [1] and animals, but its occurrence in bacteria is based more on inference rather than its isolation in a pure state [3, 7, 9, 11, 12, 13, 15].

Nutrition: general aspects

Irrespective of the organism, the continuance of life and the synthesis of cytoplasm are dependent on the availability

of the same basic materials, namely, mineral salts, water and sources of carbon and nitrogen together with a mechanism providing energy in a form that can be utilized in biological systems. Autotrophs are organisms whose carbon requirements are entirely satisfied by CO_2 (perhaps in some by CO). On the other hand, heterotrophs require a more complex carbon source, i.e. an organic compound, as well as CO_2. Moreover, heterotrophs usually derive their energy by catabolism of the organic carbon source and are therefore to be contrasted with autotrophs which obtain their energy either from light (photosynthetic autotrophs) or by the oxidation of inorganic substances, e.g. H_2S, S, $Na_2S_2O_3$, NH_4^+, NO_2^-, H_2 or Fe^{++} (chemosynthetic autotrophs). Each chemosynthetic autotroph oxidizes one specific compound, or in certain cases, a limited number of chemically related compounds, and presumably part of the energy released during these oxidations becomes available in the form of energy-rich phosphate groups. How the light energy absorbed by the chlorophyll of photosynthetic organisms becomes converted into a form that can be utilized in enzymic reactions is not yet known, though recent experiments have provided some indications of a possible mechanism [17].

All autotrophs derive their nitrogen from an inorganic source and, depending on the organism, use molecular N_2, NH_4^+, nitrate or nitrite. Although one or more of the latter may serve as a complete source of nitrogen for certain heterotrophs, the nutritional requirements of many of these organisms are not so simple. It appears that such heterotrophs are unable to synthesize one or more of the organic constituents of cytoplasm and they are therefore only able to grow if these substances are present in their environment, i.e. they are exacting towards these substances. The ability to synthesize complex organic nitrogenous compounds is especially variable, and whilst some organisms are exacting towards only one compound, e.g. *Salmonella typhosa* to tryptophan and *Proteus vulgaris* to nicotinic acid, the nutrition of other heterotrophs is far more complex, e.g. *Leuconostoc mesenteroides* P-60 requires eighteen amino-acids and

at least eleven growth factors. (The term *growth factor* is used here in the same sense as vitamin in animal nutrition.) With regard to the amount of carbon used for the synthesis of cellular material, the contribution of the organic compound serving as a source of carbon and energy varies inversely with the number of cytoplasmic constituents which the heterotroph derives preformed from the environment: in a rich medium this compound may function primarily as a source of energy. At one time, autotrophs were differentiated from heterotrophs on two counts: firstly that heterotrophs were unable to incorporate the carbon of CO_2 into organic molecules, and secondly that autotrophs live entirely and exclusively at the expense of inorganic substances. There is now adequate information to show that both of these statements require modification [18, 19, 5]. The growth of heterotrophs is in fact dependent on the presence of CO_2 and they are known to possess enzyme systems accomplishing its fixation: but, although essential, CO_2 is neither a complete nor a major source of carbon for heterotrophs. Furthermore, it has been established that several organisms regarded as autotrophs can exist heterotrophically. For example, in the presence of a suitable H-donor, the purple sulphur bacteria (Thiorhodaceae) obtain their energy from light, whilst CO_2 and NH_3 (or N_2) serve as complete sources of C and N. The H-donor may be an *inorganic* form of sulphur or an *organic* substance such as a fatty acid, and the Thiorhodaceae can therefore be regarded as facultative autotrophs. On the other hand, the green sulphur bacteria use only an inorganic H-donor and appear to be obligate autotrophs. The Athiorhodaceae (non-sulphur purple bacteria) require certain growth factors and usually an organic H-donor, i.e. they are heterotrophs, although they too derive their energy from light.

Synopsis of monograph

Many organisms can derive their energy either directly or indirectly from nitrogenous compounds, and examples of this feature of their metabolism are given in separate

chapters devoted firstly to the fermentation and oxidation of amino-acids by heterotrophs, and secondly to the autotrophic nitrifying bacteria. It will be seen that in nature the ammonia produced during the decomposition of amino-acids may suffer one of three fates: (1) oxidation by the nitrifying bacteria to nitrate (Chap. III), (2) after oxidation to nitrate, conversion to molecular N_2 and nitrous oxide (Chap. III), (3) incorporation into organic molecules (Chap. V). The anabolic aspects of nitrogen metabolism culminate in the formation of two major groups of complex substances, proteins and nucleic acids. The latter are considered in a separate chapter whilst protein synthesis is traced step by step, beginning with the mode of incorporation of nitrogen from molecular N_2 and NH_3 into organic molecules. After dealing with the synthesis of amino-acids and with the mechanisms operative in the absorption of these compounds from the environment, attention is next directed to the significance of peptides in intermediary metabolism, the problems of protein synthesis and how amino-acids become joined together by peptide bonds. This part of the monograph concludes with a chapter devoted to the enzymes responsible for proteolysis, a process which ultimately yields free amino-acids. The catabolism of the latter is discussed at the beginning of the monograph, consequently it will be appreciated that the metabolism of amino-acids and proteins has been studied at various stages in a cycle. The underlying theme of the monograph is none other than that known to all biologists as the *nitrogen cycle*, and an attempt has here been made to analyse some of the component steps of the cycle from the standpoint of the biochemistry of the various reactions and the micro-organisms concerned (Fig. 1.1).

Purely for convenience, and in order to avoid possible confusion, the microbial metabolism of nucleotides, nucleosides, purines and pyrimidines is discussed in a separate chapter. This field is now being studied intensively and there has been little time to correlate many of the experimental facts rapidly being placed at our disposal. Partly for this reason, and partly because of limitations in the amount of

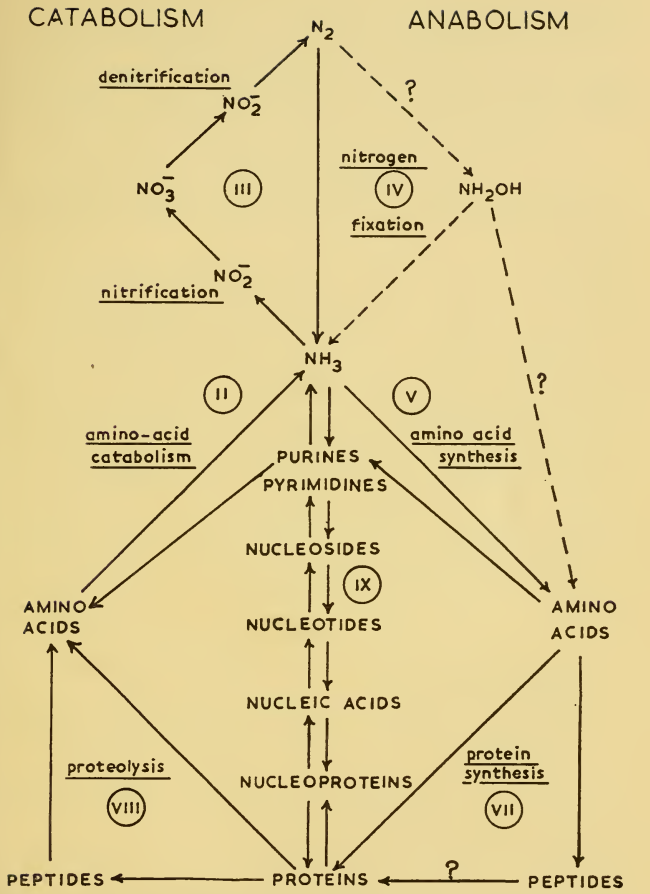

FIG. 1.1.—The Nitrogen Cycle. The roman numbers enclosed within circles denote the numbers of the chapters dealing with the various topics shown in the scheme

space available, the subject-matter of the chapter is confined to a few selected topics.

In the concluding chapter the mode of action of chemotherapeutic agents is considered in terms of their observed effects on the metabolism of compounds containing nitrogen.

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CHAPTER II

AMINO-ACID CATABOLISM

MANY heterotrophs can utilize organic nitrogen compounds, in addition to carbohydrates, as primary sources of carbon and energy. In general, the nitrogen is first removed from the compound and the product is then fermented or oxidized by the same terminal pathways that are operative in the catabolism of carbohydrates and fatty acids. Certain heterotrophs, apparently lacking the ability to metabolize exogenous sugars, are entirely dependent on organic nitrogen compounds, such as amino-acids, purines or pyrimidines, as sources of carbon and energy. Although the end-products of the catabolism of these organisms have been studied, little is yet known about the routes of their formation or the enzymes responsible for the individual steps.

The catabolism of amino-acids commences either with an oxidative deamination or with the removal of a specific group by a non-oxidative process. It is unlikely that the latter is directly responsible for making energy available to the organism, but in either case examples are known in which the further metabolism of the products proceeds by routes which result in the formation of energy-rich phosphate groups. Thus pyruvate may arise by the non-oxidative deamination of serine (p. 23) or the oxidative deamination of alanine (p. 11), and its oxidation by the pyruvic oxidase system is accompanied by the formation of energy-rich phosphate groups [39]. The first part of this chapter is concerned with mechanisms and enzymes which accomplish the oxidative catabolism of amino-acids, whilst the second part is devoted to enzyme systems whose primary mode of attack is non-oxidative.

Amino-acid oxidases

The amino-acid oxidases oxidize amino-acids to the corresponding keto acids and are specific for either the L or

the D stereo-isomers of their substrates,

$$RCH(NH_2)COOH+H_2O=RCOCOOH+NH_3+2H$$

The transfer of hydrogen from the amino-acid to a suitable acceptor, typically O_2, appears to be mediated by one or more carrier substances, and usually the enzyme has a prosthetic group capable of functioning in this manner. Enzymes of this type are the L-amino-acid oxidases of *Neurospora crassa* and *N. sitophila* [7], *Proteus vulgaris* [58], *Penicillium notatum* and *Aspergillus niger* [37]. Each of these oxidases attacks a wide variety of amino-acids, although the possibility that the observed activity is due to several very similar, but specific, enzymes has not been ruled out. Oxygen can be replaced *in vitro* by reducible dyes, such as methylene blue, or by ferricyanide. There is evidence that the enzyme from *N. crassa* possesses a prosthetic group, adenine flavindinucleotide, which enables hydrogen to be transferred directly to O_2, resulting in the formation of H_2O_2 [10]. In the presence of catalase (present in *Neurospora*), the oxidation of one gram mole of amino-acid involves the overall uptake of one gram atom of oxygen. The mycelium of *N. crassa* also contains a similar oxidase specific for D-amino-acids [7].

Whether the oxidase from *Pr. vulgaris* also has a flavin prosthetic group has not yet been established, and although one atom of oxygen is taken up per molecule of amino-acid oxidized, there is no evidence that H_2O_2 is first formed and subsequently decomposed by catalase. There must be more than one oxidase in *Pr. vulgaris* since washed suspensions oxidize more amino-acids than the cell-free enzyme preparation [58]. Oxygen is required for the deamination of glycine, alanine and glutamic acid by washed cell suspensions of *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus mycoides* [cf. 25]: using cells treated with toluene to prevent the further metabolism of pyruvate, it can be shown that the deamination of alanine by *Esch. coli* proceeds quantitatively according to the following equation:

$$CH_3CH(NH_2)COOH+\tfrac{1}{2}O_2=CH_3COCOOH+NH_3$$

but nothing is known about the properties of the enzyme concerned.

Glutamic acid dehydrogenase

The deamination of glutamic acid by *Esch. coli* is due to the enzyme L-glutamic acid dehydrogenase with the co-enzyme triphosphopyridine nucleotide (TPN) [2]: the end-product is α-ketoglutaric acid and it is believed that the reduction takes place in two stages:

$$COOH.(CH_2)_2CH(NH_2)COOH+TPN^+ \rightleftharpoons COOH.(CH_2)_2C(:NH)COOH+TPN.H+H^+$$

$$COOH.(CH_2)_2C(:NH)COOH+H_2O \rightleftharpoons COOH.(CH_2)_2COCOOH+NH_3$$

The system is reversible, with the equilibrium in favour of the synthesis of glutamic acid. A similar specific glutamic dehydrogenase occurs in *Saccharomyces cerevisiae* [1], *Clostridium sporogenes* [44], *N. crassa* [23], and probably in *Haemophilus pertussis* [34] and *H. parainfluenzae* [36]. *Haemophilus influenzae* will not grow except in the presence of a porphyrin (the X-factor) and diphosphopyridine nucleotide (DPN), TPN or nicotinamide riboside (the V-factor). The oxidative activity of cells harvested from a medium deficient in the V-factor was considerably increased by the addition of DPN or TPN, and in this way Klein has shown that the latter are involved in the oxidation of aspartic and glutamic acids to CO_2, NH_3 and acetic acid [36]. Unlike *H. parainfluenzae*, no volatile fatty acid was formed during the oxidation of amino-acids by *H. pertussis*, an organism which requires neither X nor V factors, and in the experimental conditions employed by Jebb and Tomlinson, only carbon from glutamic acid was incorporated into cell substance [34].

Oxidation of tryptophan by Pseudomonas spp.

A characteristic feature of species of *Pseudomonas* is that they possess or quickly acquire the ability to utilize any one of a wide variety of oxidizable organic substances as sources

of carbon and energy, a property which has been widely exploited by Stanier in the elucidation of metabolic pathways by the technique of 'simultaneous adaptation' [54]. If an organism exhibits little or no detectable activity against a certain substance, and if the inclusion of this substance in its environment evokes, in the absence of cell division, a marked increase in the organism's ability to metabolize that substance, then adaptation is said to have taken place. If an organism can metabolize a particular compound, the hypothesis of simultaneous adaptation postulates that it can also metabolize immediately, and at a comparable rate, any substance which is an intermediate in the metabolism of that compound (assuming that the intermediate can pass into the cells). If there is a lag period prior to the rates of utilization becoming comparable, then it may be concluded that the substance cannot be attacked by the existing metabolic systems, in other words, it is not an intermediate, and is only metabolized after adaptation has taken place. From such data it may be possible to deduce the probable route by which a substance is catabolized, but unequivocal proof requires not only direct evidence of formation of the intermediates but also isolation of the appropriate enzymes. The aerobic nature of the *Pseudomonas* spp. means that the overall catabolism of whole cells can be studied manometrically in terms of an uptake of O_2, and an example of this technique is provided by the investigations concerned with the degradation of tryptophan [55]. After being grown on, or otherwise adapted to tryptophan, some strains of *Pseudomonas* are simultaneously adapted to formylkynurenine, kynurenine, anthranilic acid and catechol; whilst others are adapted to kynurenine and kynurenic acid, but not to anthranilic acid or catechol. Work with cell-free extracts [30] revealed that the pyrrole ring of tryptophan (Fig. 2.1) is first ruptured by a peroxidase-oxidase system in which both H_2O_2 and O_2 are involved, and the product, formylkynurenine, is then hydrolysed by formylase into formic acid and kynurenine. In some strains, the pyrrole ring is now reformed, thus producing kynurenic acid, but the route by which this substance is metabolized remains

unknown. In other strains, kynurenine is split by kynureninase into alanine and anthranilic acid. The oxidation of the latter to CO_2 proceeds via catechol, *cis-cis*-muconic acid, and β-ketoadipic acid. Whilst the three enzyme systems, tryptophan peroxidase, kynureninase and pyrocatechase, were all highly active in cell-free extracts from organisms

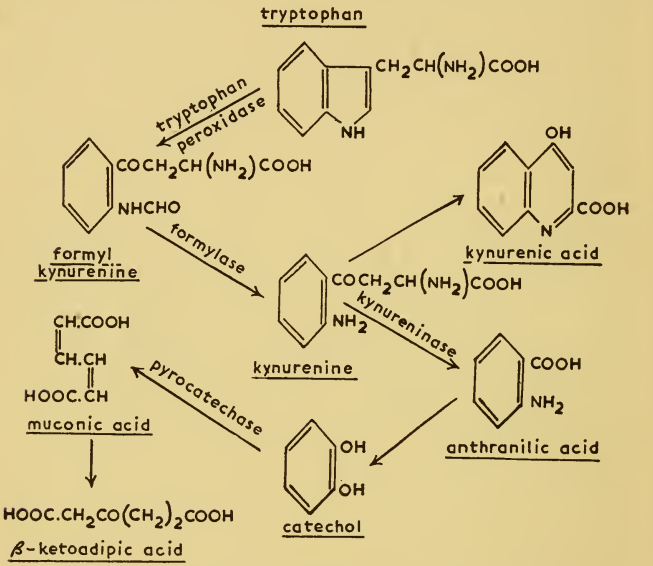


FIG. 2.1.—Pathways of tryptophan degradation in *Pseudomonas* spp.

grown in the presence of tryptophan, extracts from cells grown on asparagine exhibited negligible activity. It is notable that in none of the oxidative reactions could dyes like methylene blue replace O_2 as the H-acceptor. The degradation of tryptophan via catechol has been termed the 'aromatic pathway' whilst that by way of kynurenic acid is known as the 'quinoline pathway'. Animal tissues degrade tryptophan by the former route. The routes of tryptophan

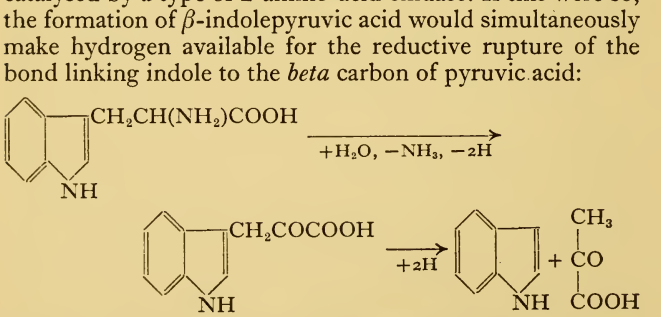
catabolism by *Bacillus subtilis*, another aerobe, appear to be similar to those found in the *Pseudomonas* spp. [40].

Tryptophanase

The appearance of indole in the culture medium is a valuable diagnostic test in bacteriology and it is readily detected by the formation of a pink compound in the presence of *p*-dimethylaminobenzaldehyde and acid. Hopkins and Cole were the first to isolate tryptophan and immediately suspected and proved that it was the natural precursor of this indole. The enzyme system concerned has been termed tryptophanase, and is found in some species of *Escherichia*, *Proteus* and *Vibrio*, though there are strain differences in any one species. Oxygen is probably not directly involved in the initial step of the tryptophanase reaction, but indole tends to accumulate only in aerobic conditions; e.g. in the absence of O_2, *Esch. coli* formed indolepropionic acid and little, if any, indole. When washed cells of *Esch. coli* were incubated with tryptophan and the system adequately aerated, the uptake of oxygen corresponded to that required for the complete oxidation of the alanine side chain [64]. Most of the experiments concerned with the mode of action of tryptophanase have been performed with preparations of *Esch. coli*, and prior to attempting to prepare the enzyme system in a cell-free state attention was directed to the factors affecting its activity in whole cells [29]. Tryptophanase was found to be adaptive, and extremely active cells were obtained from vigorously aerated media containing tryptophan. Whether *Esch. coli* grown in the absence of the substrate exhibits detectable tryptophanase activity appears to depend on the strain of the organism concerned. The inclusion of glucose in the medium may result in the suppression of indole formation, and if it does, the cells from such cultures do not exhibit tryptophanase activity. This effect is not due to growth in an acidic medium since other sugars are metabolized with the formation of acidic end-products and yet they do not suppress the adaptive formation of tryptophanase. However, several workers have reported activity in cells derived

from cultures grown in the presence of glucose and a probable explanation of their results is that tryptophanase is only developed after all or most of the glucose has been decomposed. There is evidence that this enzyme system is not developed in cultures grown in an amino-acid rich medium containing glucose because phenylalanine and tyrosine exert an inhibitory effect. The specificity of tryptophanase is high and indole is only formed from compounds related to L-tryptophan provided that the α-carboxyl and α-amino groups, the β-position in the side chain, and the N of the indole ring are unsubstituted.

Wood, Gunsalus and Umbreit [63] have obtained from *Esch. coli* a cell-free preparation which attacked tryptophan with the formation of equimolecular amounts of indole, NH_3 and pyruvic acid. Their tryptophanase preparation was activated by pyridoxal phosphate and would not deaminate either serine or alanine. They therefore concluded that neither of these amino-acids is an intermediate in the degradation of tryptophan by this route. Dawes and Happold have performed similar experiments and reached the same conclusion. Although their system produced equimolecular amounts of indole and pyruvic acid there was an excess of NH_3. Whilst no correlation with tryptophan disappearance was attempted, these observations may indicate that the initial step is one of deamination which is perhaps catalysed by a type of L-amino-acid oxidase. If this were so, the formation of β-indolepyruvic acid would simultaneously make hydrogen available for the reductive rupture of the bond linking indole to the *beta* carbon of pyruvic acid:



There is little direct evidence to support this hypothesis,

although it was noted that in addition to pyridoxal phosphate, the enzyme preparations contained riboflavin, a possible carrier of hydrogen. The bond joining indole to the side chain appears to be susceptible to reduction since indole is readily formed *in vitro* when tryptophan is either refluxed with Raney nickel and absolute alcohol containing a little HCl, or boiled with aqueous NaOH and catalytic amounts of Cu^{++} or Co^{++}. A summary of the other mechanisms which have been proposed to explain the mode of action of tryptophanase will be found in the review by Happold [29].

With the possible exception of tryptophanase, all the enzyme systems discussed above accomplish the oxidative catabolism of amino-acids in association with molecular O_2 as the ultimate and natural H-acceptor. In anaerobic organisms either amino-acids themselves or compounds derived from them may fulfil this function.

Stickland reaction

Stickland was the first to demonstrate that amino-acids take part in anaerobic oxido-reduction reactions, certain acids acting as H-donors whilst others function as H-acceptors. The original experiments were done with the strict anaerobe *Cl. sporogenes* which is capable of growing in an amino-acid medium in the absence of carbohydrates, and he suggested that the organism derived its energy from reactions of this type [56]. Nutritional studies later revealed that the organism only grew well in a medium containing adequate amounts of the amino-acids shown by Stickland to be H-acceptors and H-donors [22]. In Stickland's experiments, washed cell suspensions were incubated anaerobically with the appropriate substrates in Thunberg tubes. Hydrogen-donor amino-acids were detected by their ability to reduce methylene blue or cresyl blue to the colourless leuco form. Alanine, valine, leucine and pyruvate were all active H-donors whilst phenylalanine, aspartic and glutamic acids showed some activity, reduction of the dye being accompanied by deamination of the amino-acid. Hydrogen acceptors were detected by their ability to accept hydrogen from the leuco form of a dye of suitable redox potential

(e.g. phenosafranine and benzyl viologen, but not methylene blue) and thus restore the original dye colour. Proline, hydroxyproline and glycine were reduced by such a system, though ammonia was only formed from glycine. Incubation of alanine with proline resulted in the production of 1 mole of NH_3 per mole of alanine: none was produced from the separate amino-acids. Stickland thus demonstrated that *Cl. sporogenes* catalysed oxido-reduction reactions between pairs of amino-acids, one acid acting as a H-donor, the other as a H-acceptor. The reduction of proline resulted in opening of the ring and the formation of δ-aminovaleric acid, whilst the products derived from alanine were NH_3, CO_2 and acetic acid. When cresyl blue accepted hydrogen from alanine, 2 moles of dye were decolorized for each mole of NH_3 released, indicating that the overall oxidation of alanine involved the donation of four hydrogen atoms. Although there was no direct evidence, it seemed highly probable that pyruvate was an intermediate in the decomposition of alanine and Stickland therefore proposed that the overall reaction (*d*) represented the sum of three separate reactions (*a*, *b*, *c*):

$$CH_3CH(NH_2)COOH+H_2O=NH_3+CH_3COCOOH+2H \qquad (a)$$

$$CH_3COCOOH+H_2O=CH_3COOH+CO_2+2H \qquad (b)$$

$$4H+2NH(CH_2)_3CHCOOH=2NH_2(CH_2)_4COOH \qquad (c)$$

$$CH_3CH(NH_2)COOH+2NH(CH_2)_3CHCOOH+2H_2O$$

$$=2NH_2(CH_2)_4COOH+NH_3+CO_2+CH_3COOH \qquad (d)$$

similarly:

$$CH_3CH(NH_2)COOH+2NH_2CH_2COOH+2H_2O$$

$$=3NH_3+CO_2+3CH_3COOH$$

Clostridium botulinum [15] and all the proteolytic clostridia examined by Nisman, Raynaud and Cohen [45] were capable of performing the Stickland reaction; amongst the organisms which could not were *Cl. tetani*, *Cl. tetanomorphum*, *Cl. welchii* and *Cl. saccharobutyricum*. Apart from the substances already mentioned, histidine, serine, iso-

leucine, tyrosine, methionine, ornithine, tryptophan, phenylalanine, cysteine and ethanol also act as H-donors, whilst tryptophan, tyrosine, ornithine and arginine function as H-acceptors [33, 65]. It will be noted that some amino-acids serve both as an acceptor and as a donor, and whilst the reaction is specific for the L-isomer of H-donors [cf. 56], there is no stereochemical specificity with respect to H-acceptors [65].

If *Cl. sporogenes* is grown in the presence of glucose, it develops an active hydrogenase which enables the reducing component of the Stickland system to be replaced by molecular H_2 [33]. The reaction can then be followed in terms of an uptake of H_2 and the end-products are only those derived from the amino-acid added as the H-acceptor. In an analogous manner, and perhaps unexpectedly since the organisms are strict anaerobes, the H-acceptor part of the system can be replaced by O_2 [46]. A number of amino-acids, all H-donors in the Stickland reaction, were oxidatively deaminated by washed suspensions of *Cl. sporogenes* to the corresponding α-keto acid. The uptake of O_2 was appreciably reduced by the presence of a H-acceptor amino-acid and restored to its former value by the addition of arsenite. The latter can readily be explained since although arsenite completely inhibited H-transfer to a H-acceptor amino-acid (e.g. from leucophenosafranine to proline) it had no effect on the H-donor part of the system (e.g. the reduction of phenosafranine by alanine) [47]. Hence the first step in the Stickland reaction is probably catalysed by a type of L-amino-acid oxidase which, perhaps with the aid of one or more carriers, can transfer hydrogen to O_2, a dye or another amino-acid. In cell-free extracts, DPN but not TPN was readily reduced by H-donor acids [44], but whether reduced DPN is the natural carrier and reacts directly with H-acceptor amino-acids is not known. Pyruvate and other α-keto acids are oxidized by *Cl. sporogenes* to CO_2 and a fatty acid containing one less carbon atom by a mechanism which can lead to the formation of energy-rich phosphate groups (cf. reaction *b*):

$$CH_3COCOOH+H_3PO_4 \longrightarrow CH_3CO\sim PO_3H_2+2H+CO_2 \text{ [47]}$$

Nisman and his colleagues therefore support Stickland's concept of the reaction mechanism and they believe that reactions *a*, *b* and *c* are catalysed by an L-amino-acid oxidase [51], a keto acid oxidase [cf. 39] and an amino-acid reductase respectively. There is a complete lack of knowledge concerning the mechanism by which an amino-acid is reduced to a fatty acid (reaction *c*). Reaction *a* is inhibited by KCN and secondary octyl alcohol, *b* by iodoacetate and *c* by arsenite [47]. The growth of *Cl. sporogenes* at the expense of energy derived from amino-acids is characterized by the production of acetic acid together with isobutyric, isovaleric and optically active valeric acids, derived respectively from valine, leucine and isoleucine by the Stickland reaction. On the other hand, owing to its inhibitory effect on the Stickland reaction, the utilization of glucose as the energy source yields only acetic and butyric acids [16].

Fermentation of amino-acids by other organisms [20]

Certain organisms live anaerobically by the fermentation of one particular organic nitrogen compound whilst others, although not so specific, are restricted to the utilization of a small number of chemically related compounds. These organisms have usually been isolated by the enrichment culture technique and the anaerobic incubation of a sample of mud or soil in a medium containing an organic nitrogen compound as the major source of carbon. In the event of the organisms requiring certain growth factors, a *small* amount of yeast extract is usually included in the medium after the first transfer.

The anaerobic cocci *Diplococcus glycinophilus* [11], *Micrococcus anaerobius* and *M. variabilis* [19] are specific for glycine, and no other substance is readily metabolized unless glycine is also present. If cultures of *D. glycinophilus* are not shaken, the overall fermentation is expressed by:

$$4NH_2CH_2COOH + 2H_2O = 4NH_3 + 3CH_3COOH + 2CO_2$$

The fermentation is not a simple dismutation involving the oxidation of one molecule of glycine to CO_2 and NH_3 and the reduction of three molecules to acetic acid and NH_3.

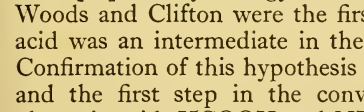
Experiments with isotopically labelled glycine have shown that most of the methylene carbon appeared as acetic acid whilst the carboxyl carbon appeared as CO_2 [4].

Alanine, serine or threonine serve as sole sources of carbon and energy for *Cl. propionicum* and are fermented to CO_2, NH_3 and fatty acids [11, 12]:

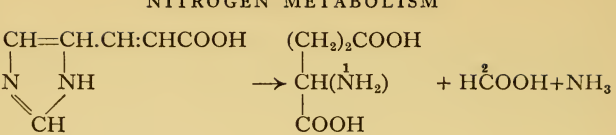
$$3CH_3CH(NH_2)COOH+2H_2O=$$
$$2CH_3CH_2COOH+CH_3COOH+CO_2+3NH_3$$
$$3CH_2(OH)CH(NH_2)COOH+H_2O=$$
$$CH_3CH_2COOH+2CH_3COOH+2CO_2+3NH_3$$

The mechanism of alanine fermentation may be comparable with that proposed for the Stickland reaction, alanine acting both as the H-acceptor and as the H-donor. Lactate and pyruvate are fermented in a similar manner and these fermentations are at least superficially comparable to the fermentation of lactate by the propionibacteria. However, whilst in the latter propionic acid arises by the decarboxylation of succinate, in *Cl. propionicum* it is probably formed from acrylate [35]. Barker and Wiken have concluded that acetate is not an intermediate in the fermentation of threonine to butyric and propionic acids, and that butyric acid probably arises directly from a C_4-compound (α-ketobutyrate?) [5].

Unlike *D. glycinophilus* and *Cl. propionicum*, *Cl. tetanomorphum* ferments glucose as well as certain amino-acids. The end-products of both glutamic acid and histidine fermentations include H_2, CO_2, NH_3, acetic and butyric acids [66], and by analogy with Edlbacher's work with liver, Woods and Clifton were the first to suggest that glutamic acid was an intermediate in the fermentation of histidine. Confirmation of this hypothesis has been recently obtained and the first step in the conversion of histidine (I) to glutamic acid, HCOOH and NH_3 involves deamination to urocanic acid (II) [60].



(I)



(II)

Urocanic acid and glutamic acid are also intermediates in the oxidation of histidine by *Ps. fluorescens*, and isotopes have been used to show that the amino-nitrogen of glutamic acid and the carbon of HCOOH are derived from the N and C in positions 1 and 2 of the imidazole ring [59]. The fermentations of *Cl. tetani* [48*b*] and *Cl. cochlearum* [3] resemble those of *Cl. tetanomorphum*. The former ferments a number of amino-acids, only one of which, histidine, is among those essential for growth; aspartic acid and serine give rise to alcohols as well as to fatty acids. It is well known that yeasts fermenting carbohydrates in the presence of amino-acids produce a number of the higher aliphatic alcohols (fusel oil), and Ehrlich showed that the latter contain one less carbon atom than the amino-acids from which they were derived, the overall reaction being:

$$R.CH(NH_2)COOH+H_2O=R.CH_2OH+NH_3+CO_2$$

Such a process may be the means whereby the nitrogen of the amino-acid is made available in the form of NH_3, but the details of the mechanism are unknown. Most of the alcohols are formed after the amino-acids have disappeared from the medium, indicating that there are intermediate stages between deamination and production of the alcohol [13].

Anaerobic α-deaminases

A characteristic feature of the anaerobic α-deaminases is that the reaction product is unsaturated and therefore such enzymes are sometimes described as desaturases. One typical example has already been mentioned, namely, the enzyme forming urocanic acid from histidine, and there is evidence that it also occurs in certain strains of *Esch. coli*, *Salmonella paratyphi* and *Shigella paradysenteriae* [50]. A similar type of reaction is catalysed by aspartase, an enzyme

found in *Esch. coli*, *Ps. fluorescens*, *Serratia marcescens*, *Pr. vulgaris*, *Lb. casei*, and perhaps in yeasts [67, 17, cf. 21]:

$$COOH.CH_2CH(NH_2)COOH \rightleftharpoons COOH.CH{:}CH.COOH+NH_3$$

The system is reversible and by using cells treated with cyclohexanol to prevent conversion of the fumarate to succinate or malate, it can be shown that the equilibrium favours the synthesis of L-aspartic acid [67]. Serine and threonine are deaminated anaerobically to pyruvic and α-ketobutyric acid respectively, though whether one deaminase catalyses both reactions is not yet known. Unlike aspartase, the reaction does not appear to be reversible and the first step is thought to be the removal of the elements of water, followed by the spontaneous hydrolysis of the resulting imino-compound [14*a*]:

$$CH_2(OH)CH(NH_2)COOH \xrightarrow{-H_2O} (CH_2{:}C(NH_2)COOH) \longrightarrow CH_3C(:NH)COOH$$

$$CH_3C(:NH)COOH \xrightarrow{+H_2O} CH_3COCOOH+NH_3$$

The dehydration step is analogous to that catalysed by enolase, and cysteine desulphurase may likewise be regarded as first removing the elements of H_2S:

$$CH_2(SH)CH(NH_2)COOH+H_2O=CH_3COCOOH+H_2S+NH_3$$

Serine and threonine deaminase activity is found in *Esch. coli*, *Cl. welchii*, *Ps. pyocyanea*, *Proteus OX–19* and staphylococci: cysteine desulphurase occurs in *Sac. cerevisiae*, *Esch. coli*, *Pr. vulgaris*, *B. subtilis* and *Propionibacterium pentoseaceum* [24].

Aspartase, cysteine desulphurase, and the serine and threonine deaminases are all alike in that their activity is dependent on the presence of certain co-factors, the identity of which has not yet been completely established. For example, there was a marked reduction in the aspartase activity of washed cell suspensions of *Esch. coli* after they had been kept standing in water or buffer. This decay in activity could be prevented by the addition of a small amount of either adenylic acid (AMP) or orthophosphate, together

with a reducing agent such as cysteine, lactate or formate [28, 25]. Similarly, a loss of serine deaminase activity was prevented by glutathione, formate or AMP. During investigations of the nutrition of lactobacilli and *Strep. faecalis*, organisms exacting towards aspartic acid, Stokes, Larsen and Gunness observed that they grew well in the absence of this amino-acid provided the medium contained adequate amounts of biotin. They therefore suggested that biotin plays an important role in aspartic acid metabolism, and perhaps especially in the aspartase system, although they were unable to demonstrate the presence of the latter in these organisms [57].

Meanwhile Lichstein and his colleagues were studying the catabolism of aspartic acid and serine by *Esch. coli*, *Pr. vulgaris* and *Bacterium cadaveris*. They found that of the known B vitamins only biotin was capable of reactivating washed cell suspensions whose deaminase activity had been reduced by standing for 30 minutes in molar phosphate buffer, pH 4 [42]. This loss of deaminase activity and subsequent reactivation by biotin or by much larger concentrations of AMP was only exhibited by cells which had been grown in the presence of a yeast extract. A concentrate of compounds containing biotin was prepared from yeast and was found to be a hundred times more effective in enhancing the aspartase activity of 'aged' suspensions than was to be expected on the basis of its biotin content. Moreover, when fresh, a partially resolved cell-free preparation of aspartase was activated either by yeast extract or by biotin plus adenylic acid, but after standing at O° C., only the former was effective [41]. A bound form of biotin, termed biocytin, has been isolated recently from yeast, crystallized and shown to be ε-N-biotinyl-L-lysine [69]. Biocytin itself is probably not the natural coenzyme, since, unlike the impure concentrates, its activity is only comparable to that produced by an equivalent amount of free biotin [68]. Wood and Gunsalus have prepared from *Esch. coli* a purified cell-free system possessing serine and threonine deaminase activity, and although both adenylic acid and glutathione were required, biotin did not appear to be necessary [62].

Furthermore, Binkley's dialysed cell-free preparation of serine deaminase was reactivated by Zn^{++} [8]. In contrast with this work with bacteria, it is now clear that *Neurospora* possesses two deaminases—one specific for the L isomers of threonine and serine [71], the other for the D isomers [70] —and that both of these enzymes are activated by pyridoxal phosphate. Biotin, AMP and GSH, either separately or together, did not affect the activity of cell-free preparations. Though the D-serine deaminase of *Esch. coli* is likewise activated by pyridoxal phosphate, attempts to show that the corresponding L-serine deaminase is activated by this substance have been unsuccessful [43*b*]. Pyridoxal phosphate is also reported to be the activator of the cysteine desulphurase of *Proteus vulgaris* [48*a*]. In view of these diverse observations, it is not yet possible to define the cofactors naturally associated with the anaerobic deaminases. The confusion in this field has recently been increased since a substance produced by heating glucose with acid under pressure was found to be as active as yeast extract in the activation of the resolved aspartase system of *Bact. cadaveris* and yet did not contain biotin [14*b*]. Winzler, Burk and du Vigneaud [61] have observed that unless biotin was added to the system, washed cells of biotin-deficient *Sac. cerevisiae* were incapable of assimilating exogenous NH_4^+. Their observation can be readily explained, if, as Lichstein's work indicates, this growth factor is an essential component of aspartase, one of the systems by which inorganic nitrogen is incorporated into organic molecules (p. 60).

In general, the anaerobic deaminases are most active in cells harvested from cultures at the cessation of active cell-division. Moreover, the presence of O_2 favours the development of the aerobic amino-acid oxidases whilst anaerobic conditions favour the anaerobic deaminases. The metabolic quotients of the former (Q_{NH_3} about 30) are very much lower than those of the latter (Q_{NH_3}=200–1,000). After growth in the presence of glucose, organisms usually possess poor deaminase activity, and although in general the optimum pH for deaminase activity is in the range 8–10, this effect cannot be explained in terms of growth in an

acidic environment since activity is not increased by buffering the medium. Nor must it be assumed that glucose is used preferentially as a source of carbon and energy and that in consequence the organisms tend not to synthesize the deaminases. On the contrary, the reduced activity may be a reflection of the lack of essential co-factors since Boyd and Lichstein [9] found that the low serine deaminase activity of a washed suspension of *Esch. coli* (grown in the presence of glucose) was almost immediately increased by the addition of biotin, adenylic acid, yeast extract or liver extract.

Having considered the enzymes which deaminate amino-acids, it is now convenient to deal with those which attack other groups in the molecule.

Arginine dihydrolase

Washed suspensions of *Strep. faecalis*, *Staph. aureus*, *Cl. septicum* and *Cl. sporogenes* decompose arginine into ornithine, NH_3 and CO_2:

$$\begin{matrix} NH_2 \\ \diagdown \\ \quad C.NH(CH_2)_3CH(NH_2)COOH \\ // \\ NH \end{matrix} + 2H_2O$$

$$= 2NH_3 + CO_2 + NH_2(CH_2)_3CH(NH_2)COOH$$

Since urea is not attacked by *Strep. faecalis*, the overall reaction cannot be explained in terms of the splitting of arginine into ornithine and urea by arginase and the subsequent decomposition of urea by urease. Hills [32] therefore proposed that the enzyme system in *Strep. faecalis* and *Staph. aureus* should be known as arginine dihydrolase. Recent work has shown that more than one enzyme is involved. The first step in the reaction is the formation of ammonia and citrulline, $NH_2CONH(CH_2)_3CH(NH_2)COOH$, which is then degraded to ornithine by an enzyme system activated by inorganic phosphate, Mg^{++} or Mn^{++}, and ATP or AMP [38, 52]. *Strep. faecalis* is exacting towards arginine, and arginine can be replaced by ornithine only if the medium also contains adequate amounts of CO_2. Carbon

dioxide is not produced during the fermentation of glucose by *Strep. faecalis*, and since growth is not possible in its absence Gale has suggested that arginine dihydrolase is a mechanism whereby this metabolite is made available [26]. He found that strains with high arginine dihydrolase activity grew better if the amount of arginine initially present in the medium was such that it was not all decomposed by the time the pH became unfavourable for further growth. Alternatively, it is feasible that arginine serves as a source of energy and that arginine dihydrolase activity is connected with the organism's energy metabolism, since Knivett [38] has shown that the conversion of citrulline to ornithine is accompanied by the phosphorylation of ADP to ATP.

Amino-acid decarboxylases

Although for several years bacteria have been known to form amines from amino-acids, no study of the enzymes concerned was made until the work of Gale [27]. The amines arise by decarboxylation of α-amino-acids:

$$RCH(NH_2)COOH = RCH_2NH_2 + CO_2$$

and the initial experiments demonstrated the existence of six amino-acid decarboxylases, specific respectively for the L-isomers of tyrosine, lysine, ornithine, arginine, histidine and glutamic acid. They have been found in the genera *Escherichia*, *Streptococcus*, *Clostridium*, *Proteus* and *Lactobacillus*. Whilst an organism may possess more than one decarboxylase, the distribution of the enzymes amongst the strains of any one species is variable. These enzymes are active in the pH range 2–6 and have a sharp optimum. In general the substrates possess an α-amino and an α-carboxyl group together with another polar group at the opposite end of the molecule. Substitution in any of these groups yields substances which are not attacked and the introduction of a hydroxyl group in another part of the molecule is the only known structural modification which does not affect susceptibility to decarboxylation, e.g. β-hydroxyglutamic acid, β-hydroxylysine and 3:4-dihydroxyphenylalanine are decarboxylated by the glutamic, lysine and tyrosine decarboxylases

respectively. Because of their specificity and the fact that decarboxylation proceeds to completion, these enzymes are invaluable for the quantitative determination of the corresponding amino-acids. The analytical procedure originated by Gale is based on the use of a washed suspension or an acetone powder of the appropriate organism and the manometric determination of the CO_2 released.

The growth conditions are especially important in determining whether the cells develop highly active decarboxylases, and owing to their adaptive nature, the medium must contain the specific substrate. The enzymes are not formed unless growth occurs in an acid environment, and this is usually attained by allowing the organisms to metabolize glucose. Temperature is also important: for example, the decarboxylase activity of *Esch. coli* is better when cultures are grown at 20–26° C. rather than at 37° C., but the opposite is true of *Strep. faecalis* and *Cl. welchii*. Decarboxylase activity becomes maximal at the cessation of active cell division. Except for the histidine enzyme, there is evidence that all the amino-acid decarboxylases possess a prosthetic group, codecarboxylase, the existence of which was first discovered by Gale during the purification of the lysine decarboxylase. Fractionation with $(NH_4)_2SO_4$ in alkaline conditions resulted in the precipitation of an inactive apoenzyme to which decarboxylase activity could be restored by the addition of extracts of bacteria, yeast or animal tissues. Gunsalus and his co-workers later identified codecarboxylase as being pyridoxal phosphate. It is therefore essential that the growth medium should contain adequate amounts of pyridoxin, since even when the organism is not exacting towards this factor, the rate of synthesis may be insufficient to saturate the apoenzyme. The apoenzyme of tyrosine decarboxylase can be prepared [6] by growing *Strep. faecalis* in a pyridoxin-deficient medium containing D-alanine, a substance which, according to the strain, replaces or reduces the organism's pyridoxin requirements. Washed cells of bacteria grown in this way exhibit no activity unless the experimental system contains either synthetic pyridoxal phosphate, pyridoxal or natural code-

carboxylase, and they can therefore be used for assaying codecarboxylase.

More recent work has shown that the tyrosine decarboxylase will also attack phenylalanine [43*a*] and *m*-tyrosine [53], and that *Esch. coli* possesses a specific enzyme which decarboxylates α,ε-diaminopimelic acid to lysine. Unlike the other decarboxylases, the activity of the latter is high even in cells grown in the absence of the substrate, and furthermore there is evidence that the reaction is reversible: like the other decarboxylases the prosthetic group appears to be pyridoxal phosphate [18]. Cultures of *Proteus* spp. grown in an amino-acid medium have been found to contain amines derived from the branched chain amino-acids valine, leucine and isoleucine, but their mode of formation is not yet known [49].

Fermentations usually give rise to acidic end-products, consequently as the fermentation proceeds, the pH of the medium eventually reaches a value below which no further growth is possible. The highly basic amines formed as the result of decarboxylase activity tend to counteract this fall in pH, and Gale has therefore proposed that the decarboxylases may be regarded as a type of 'neutralization mechanism' [27]. Since the partial pressure of CO_2 in an acidic medium is low, another possible function of the decarboxylases is to make this essential metabolite available inside the cell. The observation that *H. parainfluenzae* is exacting towards putrescine, the amine formed by the decarboxylation of ornithine, implies that the amines themselves may be of some significance in intermediary metabolism [31].

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CHAPTER III

NITRIFICATION AND DENITRIFICATION

Nitrification

Nitrosomonas and *Nitrobacter* are two genera of strictly aerobic chemosynthetic autotrophs which respectively obtain their energy by the oxidation of ammonia to nitrite (NO_2^-), and nitrite to nitrate (NO_3^-).

$$\textit{Nitrosomonas},\ NH_3+1\tfrac{1}{2}O_2=HNO_2+H_2O$$
$$\textit{Nitrobacter},\quad NO_2^-+\tfrac{1}{2}O_2=NO_3^-$$

These organisms play an important role in the formation of NO_3^- from NH_3 and organic nitrogen compounds, a process occurring in soil and the beds of sewage purification works [7] and known as nitrification. Most organic nitrogen compounds are only nitrified by *Nitrosomonas* and *Nitrobacter* after they have been degraded by heterotrophs to NH_3.

The first evidence in favour of Pasteur's suggestion that nitrification was due to micro-organisms came from the classic experiments of Schloessing and Muntz [27]. After sewage effluent had percolated through a column of sand and chalk for about twenty days, they noted that NH_3 was being converted almost quantitatively into NO_3^-. This conversion was completely stopped if the column was subjected to conditions injurious to life, e.g. heat or chloroform, but commenced again after washing with non-sterile water derived from soil. Of the organisms known at that time, none could oxidize NH_3 to NO_3^-, and the first attempts to isolate the causative agents by plating soil on nutrient gelatin media all ended in complete failure. The lack of success was traced to the inhibitory effect of organic substances on the growth of nitrifying organisms and resulted in the introduction of media containing only inorganic

salts [33]. After inoculating such media with soil, Winogradsky readily obtained excellent growth accompanied by nitrification, and the cultures were easily maintained through successive transfers in a mineral medium containing potassium phosphate, NH_4Cl, $MgSO_4$ and K_2CO_3. Using the enrichment culture technique, the isolation of organisms oxidizing NH_3 was facilitated by repeated subculture in an inorganic medium containing NH_4^+ but no added NO_2^-: similarly, a medium containing NO_2^- and no NH_4^+ was used for organisms oxidizing NO_2^-. Winogradsky solved the problem of how to obtain discrete colonies on a solid yet completely inorganic medium by using silica gel as the matrix for the mineral salts [34]. The colonies are minute (diam. 200 μ) and micro-manipulators are sometimes used to pick out and transfer those required for inoculating subcultures. Winogradsky was the first to obtain indisputably pure cultures of organisms which specifically oxidized either NH_3 (*Nitrosomonas europaea*) or NO_2^- (*Nitrobacter winogradsky*). About the same time, Warington [31] and the Franklands [8], working independently, obtained cultures which oxidized NH_3 to NO_2^- and which appeared to be pure as judged by microscopic examination and the absence of growth on gelatin plates. The isolation of pure cultures of the nitrifiers is difficult because their slow growth favours the appearance of heterotrophs which grow rapidly even if only traces of organic matter are present in the medium. Great care is therefore required in assessing the purity of any culture of nitrifying bacteria and no growth should be detectable after incubating plates of nutrient agar streaked with such cultures. Lees has found that adequate aeration of the medium greatly facilitates the isolation and culturing of *Nitrosomonas* [14].

Some strains of the nitrifiers are actively motile whilst others appear to be habitually associated with surfaces, e.g. they readily adhere to granules of $CaCO_3$ [cf. 19]. Many of these organisms prefer a slightly alkaline environment [27, 18], and for this reason $CaCO_3$, K_2CO_3 or $MgCO_3$ are frequently added to the medium [34, 20, 12]. Such substances

serve as a source of CO_2 and at the same time prevent the H^+ produced during nitrification from lowering the pH to a value unsuitable for growth [20]. The optimum pH for the growth of a particular strain tends to be related to the pH of the soil from which it was isolated, e.g. one from a peaty soil will nitrify in relatively acid conditions [11]. Meyerhof observed that the growth of *Nitrosomonas* and *Nitrobacter* is inhibited by high concentrations of their respective substrates. Furthermore, high concentrations of NH_4^+ also inhibited the oxidation of NO_2^-, and the higher the pH of the medium the greater the inhibition, both of growth and of nitrite oxidation. Both NH_4^+ and NO_2^- in excessive concentrations are known to be injurious to cells in general, and the effect of pH may be explicable on the basis that conditions of high pH favour the formation of unionized NH_3 which may enter the cells more easily than the ammonium ion [20].

The autotrophic nature of the nitrifiers, together with their apparent inability to grow in organic media, led Winogradsky to conclude that they neither required nor utilized organic nutrients. There has been much controversy as to whether all organic materials are toxic to these organisms and whether they can in fact assimilate at least some of these substances. There is now a certain amount of evidence that their growth is stimulated by small amounts of peptone, by yeast extracts and by a partial hydrolysate of egg albumin [9]: moreover, some strains of *Nitrosomonas* can grow in the presence of high concentrations of glucose (10%) [11, cf. 12]. Some evidence that the nitrifiers may be able to obtain energy in a manner typical of heterotrophs, namely by the degradation of organic compounds, has been obtained by Bömeke. In manometric experiments with thick suspensions of *Nitrosomonas* and *Nitrobacter*, he found that in the absence of exogenous nutrients, these organisms absorbed O_2, indicating that they could obtain energy by the oxidation of endogenous reserve materials [6]. Whilst there have been many experiments purporting to show that organic media support the growth of the nitrifiers, the majority of

these investigations have been discounted on the grounds that the cultures were contaminated with heterotrophs [35,

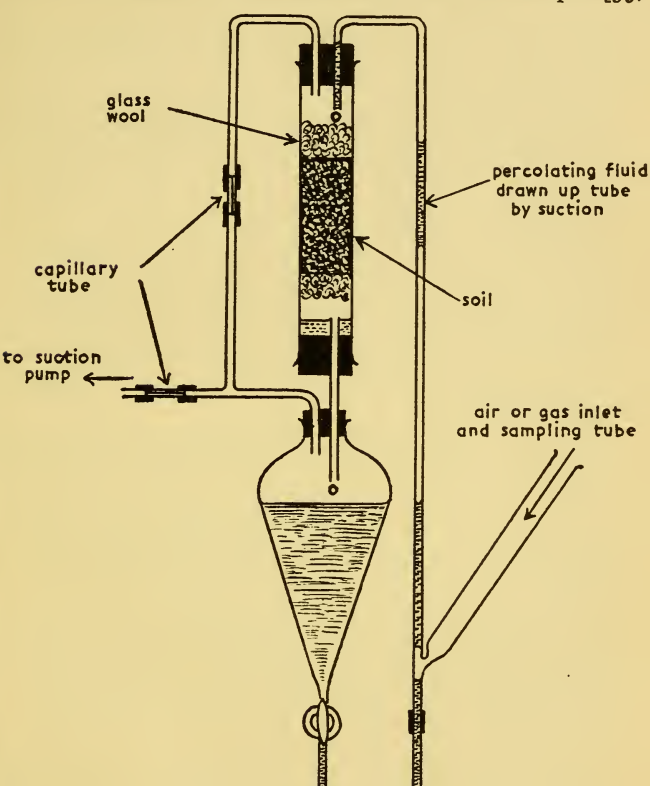


FIG. 3.1.—Lees' soil percolation apparatus, as modified by Audus [3]

12]. Quastel and Scholefield have pointed out that organic inhibitors fall into two groups, those which inhibit in concentrations of 0·01 M. or less and those which inhibit only

in very high concentrations [22]. But it must also be emphasized that the inhibitory action of any substance is determined by the organism concerned and also by the physical properties of the environment, e.g. peptone is far less inhibitory in the presence of sand than in ordinary liquid cultures [32].

Owing to their slow growth and the difficulty of isolating pure cultures, there have been few comprehensive investigations of the metabolism of *Nitrosomonas* and *Nitrobacter*. Meyerhof has dealt with the effect of substrate concentration, inorganic ions and various inhibitors on the rate of nitrification in liquid cultures [20]. Nitrification under controlled conditions in soil has been studied more recently by Lees and Quastel using a technique based on the continuous repercolation of fluid through a column of soil [16]. This was accomplished by a simple apparatus which ensured that the soil was nearly saturated with water and was adequately aerated (cf. Fig. 3.1).

When NH_4^+ was added to the percolating fluid there was a lag period after which the rate of nitrate formation gradually increased and at the end of five days became linear. During the first two days small quantities of NO_2^- were detected. A graph of the progress of nitrification is reminiscent of that expressing the rate of growth of a bacterial culture and it appears that the soil ultimately becomes saturated with nitrifying bacteria. At this stage the behaviour of the system is analogous to that of a washed cell suspension, i.e. it is metabolically active but the organisms are not actively dividing. When a fresh solution containing NH_4^+ was percolated through such a 'saturated soil', nitrification occurred at a linear rate from the beginning (Fig. 3.2). Consequently when the 'saturated soil' is treated with a substance which is nitrified directly, NO_3^- formation should commence at once and at a maximal and linear rate. A lag period implies that the substance can only be nitrified after being converted into another compound and that time is required for the formation of the appropriate adaptive enzymes in the existing bacteria or for the growth of new

organisms. For example, NO_2^- is immediately nitrified by soil which has been previously percolated with NH_4^+, but there is a lag in the nitrification of NH_4^+ by soil previously treated with NO_2^-. Hence the 'saturated soil' technique is of value in testing substances believed to be intermediates in the nitrification process. Thus hydroxylamine (NH_2OH) has long been postulated as a possible intermediate in the conversion of NH_3 to HNO_2, but there is little direct

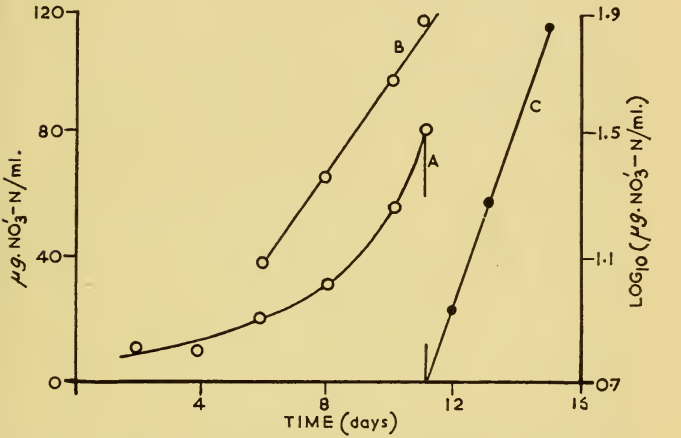


FIG. 3.2.—Course of nitrification in soil percolated with 0·01 M.-NH_4Cl: A, first percolation of fresh soil; B and C, linear relationship between time and $\log_{10}$ μg. NO_3^--N/ml. formed respectively in the first (B) and second (C) percolations [22]

evidence [15]. Free NH_2OH is toxic to bacteria and soon stops nitrification. However, when combined, as in pyruvic oxime, it is nitrified by soil enriched with nitrifiers, but only after a lag. This is due to the development of heterotrophs, species of *Archromobacter* and *Corynebacteria*, which convert the NH_2OH into NO_2^- without the intermediate formation of NH_3 [23]. The percolation technique is also valuable for determining the effects of various substances on nitrification under conditions simulating those found in

nature. Most natural organic compounds, such as amino-acids, are not inhibitory, methionine being a notable exception. Although potassium chlorate in low concentrations (e.g. 10^{-6} M.) prevents the proliferation of *Nitrobacter*, it did not affect either the growth of *Nitrosomonas* or the oxidation of NO_2^- by an established culture of *Nitrobacter*. Chloromycetin, an antibiotic containing a nitro group, is especially active against organisms oxidizing NO_2^- [22].

The rate of nitrification was found to be a function of the amount of NH_4^+ adsorbed by the base exchange complexes in the soil and could be increased by increasing the soil's base exchange capacity. No such effect was produced by the addition of materials (e.g. sand) whose only effect was to increase the available surface area. Moreover, the presence of ions such as Ca^{++}, which can displace NH_4^+, depressed the rate of nitrification. Few bacteria were found in the percolating fluid itself, and Lees and Quastel deduced that the nitrifying organisms grow on the surface of soil particles [cf. 16] around receptor areas which combine with or adsorb NH_4^+. All these areas are occupied in a 'saturated soil' and further growth of the bacteria is restricted to replacing dead cells [16]. Quastel and Scholefield have developed a technique whereby the Warburg manometer can be used in studies of soil metabolism and they showed that following the addition of NH_4^+ or NO_2^- to soil saturated with nitrifying bacteria, the O_2 uptake was equal to that required for complete oxidation to NO_3^- [22].

Nothing is known about the mechanisms which enable the organisms to utilize the energy made available by the oxidation of NH_3 and NO_2^-. Ammonia is oxidized even when the cells are unable to grow, e.g. in the absence of CO_2 [21*c*], and the suggestion has been made that a metal-activated enzyme may be involved in this process [15]. With regard to energy relationships and the efficiency of energy utilization, the best data are those of Baas-Becking and Parks, who calculated the changes in free energy (ΔF) taking place at 25° C. in conditions shown by Meyer-

hof [4] to be optimal for nitrification (NH_4^+=0·005 M.; $H^+=10^{-8}$ M.; NO_2^-=3·03 M.).

$$NH_4^+ + 1\tfrac{1}{2}O_2 = NO_2^- + H_2O + 2H^+, \quad \Delta F = -66{\cdot}5 \text{ kg. cal.}$$

$$NO_2^- + \tfrac{1}{2}O_2 = NO_3^-, \quad \Delta F = -17{\cdot}5 \text{ kg. cal.}$$

The amount of carbon assimilated as the result of the oxidation of a known amount of NH_4^+ or NO_2^- is determined experimentally, and assuming that the reduction of 1 gram mole of CO_2 to CH_2O (the generalized formula for cell substance) requires 118 kg. cal. of free energy, it can be calculated that the energy released in the oxidations is used with an efficiency of 5·9% by *Nitrosomonas* and 7·8% by *Nitrobacter* [4]. These values are only approximate, and in the case of *Nitrosomonas* it is known that the efficiency falls as the culture grows older [10].

Reduction of nitrate: denitrification

In contrast to the limited number of organisms capable of oxidizing ammonia and nitrite, several species accomplish the reverse process, namely, the *reduction* of nitrate and nitrite to ammonia, nitrous oxide (N_2O) and molecular nitrogen. Whilst ammonia may be retained in the organism or in the medium, gaseous products such as N_2 and N_2O pass readily into the atmosphere with the result that the overall nitrogen content of the organism's immediate environment is decreased. In the latter instance the biological reduction of NO_3^- and NO_2^- is often termed denitrification. Examples of organisms known to reduce NO_3^- include *Ps. fluorescens*, *Ps. denitrificans*, *Ps. stutzeri*, *Micrococcus denitrificans*, various spore-forming bacilli, *Thiobacillus denitrificans*, *N. crassa*, *Hansenula anomala*, *Azotobacter agilis*, *Esch. coli* and *Cl. welchii*. The latter five species appear to be incapable of taking the reduction as far as N_2O or N_2.

With some organisms, NO_3^- and NO_2^- serve as sources of nitrogen for the synthesis of organic nitrogen compounds: alternatively, or in addition, they may function as H-acceptors in reactions concerned with the organism's energy

metabolism. Since it is generally believed that only inorganic nitrogen in the forms of NH_3 can be incorporated into organic molecules, it is probable that in both cases the metabolic pathways have at least the initial steps in common. The end-result is different in that one leads to the assimilation of nitrogen, whilst in the other the products are excreted into the medium. The ability to reduce NO_3^- enables certain organisms to grow anaerobically in media which would otherwise only support their growth in the presence of O_2, and in such cases NO_3^- may be regarded as replacing oxygen as the ultimate acceptor of metabolic hydrogen [25]. For example, *Esch. coli* cannot grow anaerobically on lactic acid as the sole source of carbon unless the medium also contains a suitable H-acceptor, and nitrate is only one of several substances which can fulfil this function. *Serratia marcescens* and *Pr. vulgaris* behave similarly [2, 4], but other organisms are known which specifically use NO_3^- and are unable to grow anaerobically in its absence, even though the medium contains NH_3. In anaerobic conditions, the chemosynthetic autotroph, *Thio. denitrificans*, can obtain energy only by the oxidation of sulphur compounds at the expense of reducing NO_3^-:

$$6KNO_3+5S+2H_2O=K_2SO_4+4KHSO_4+3N_2+\text{energy}$$

A number of aerobic spore-forming bacilli related to *Bacillus subtilis* can live anaerobically only in the presence of NO_3^-, and they have been isolated from anaerobic enrichment cultures in media containing a high concentration of KNO_3 (8–10 per cent) [5, 30].

The first step in the reduction of NO_3^- involves its conversion to NO_2^- by an enzyme system which is adaptive in nature, and is known as nitratase [29]. The nitratase of *Neurospora* has a prosthetic group of adenine flavin dinucleotide and will use reduced TPN to reduce nitrate to nitrite [21*b*]. Some strains of *Esch. coli* are unable to reduce NO_2^- any further [24], but others reduce both NO_3^- and NO_2^- quantitatively to NH_3 in the presence of a suitable H-donor such as glucose [2, 36]. Organisms able to reduce

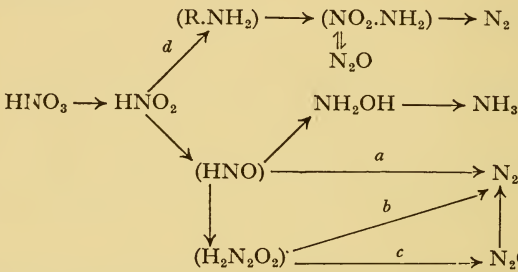
NO_3^- and which also possess hydrogenase can use molecular hydrogen as a H-donor in these reductions [28]. Woods [36] thus demonstrated that washed suspensions of *Cl. welchii* reduced NO_3^-, NO_2^- and NH_2OH to NH_3, the H_2 uptake being in accordance with the following equations:

$$NH_2OH+H_2 = NH_3+H_2O$$
$$HNO_2+3H_2 = NH_3+2H_2O$$
$$HNO_3+4H_2 = NH_3+3H_2O$$

The H_2 uptake in the third equation is that expected on theoretical grounds if NH_2OH and NO_2^- are in fact intermediates in the reduction of NO_3^-. During the early stages of the reduction of NO_3^-, a transient accumulation of NO_2^- was observed.

The recent investigations of Verhoeven [30] have done much to confirm and extend the observations and hypotheses of earlier workers in this field [13]. He found that the reduction of NO_3^- by the aerobic spore-forming bacilli resulted in the production of NO_2^-, N_2 and sometimes NH_3; NO_3^- could be replaced as the H-acceptor by NO_2^- or by N_2O. Strains producing large amounts of ammonia did not form appreciable amounts of gaseous end-products, and the converse was also true. A detailed study with one strain demonstrated that the reduction process took place in two stages. During the first stage NO_3^- was converted to NO_2^- and then to N_2O; whilst in the second, the gas evolved was mainly N_2, indicating that N_2O is the precursor of N_2. On two occasions, Verhoeven detected NH_2OH in denitrifying cultures, and thus provided some evidence in support of the contention of Blom that this compound is an intermediate in the reduction of NO_3^-. Working with *Ps. stutzeri*, Allen and van Niel have come to the conclusion that, at least in this organism, although N_2O was reduced, it was not a natural intermediate. They believe that nitramide ($NO_2.NH_2$) is a possible intermediate since N_2 was formed from a preparation of this compound but not from hyponitrous acid [1]. In the presence of a H-donor, cell-free extracts of *Ps. stutzeri* and *B. subtilis* reduce nitrate to N_2 (*Ps.*

stutzeri) or appreciable quantities of N_2O (*B. subtilis*). Extracts of both organisms convert nitrite to nitric oxide and nitric oxide to N_2 [21*a*]. Further advances in the elucidation of the pathways of the biological reduction of nitrate await the collection of more information concerning the chemical properties of nitroxyl (HNO), hyponitrous acid ($H_2N_2O_2$), nitramide and other similar compounds of nitrogen, and the development of unequivocal methods for their identification and estimation. The scheme given below is based on those proposed by Kluyver [cf. 30] and by Allen and van Niel: it will be realized that there is no *direct* evidence that nitroxyl or hyponitrous acid or nitramide is a *natural* intermediate.



Hypothetical Pathways of Nitrate Reduction in Micro-organisms

Routes '*a*' and '*b*' may be operative in *Ps. denitrificans*, and '*c*' in the aerobic spore-forming bacilli [30]. Allen and van Niel postulate that in *Ps. stutzeri*, NO_2^- enters into organic combination and is reduced to an amino compound ($R.NH_2$) which then reacts with another substance, perhaps NO_2^- itself, to yield nitramide (route '*d*').

The presence of oxygen tends to suppress the reduction of NO_3^-, the degree of inhibition being determined both by the partial pressure of O_2 and the organism concerned, e.g. *Esch. coli* [29] is more sensitive than *Ps. denitrificans* [26]. It is interesting to note that restricted aeration of cultures of the denitrifying bacilli resulted in the production of large amounts of NH_3, even by those strains which in anaerobic

conditions produced large amounts of N_2 and N_2O. However, no such effect was obtained with *Pseudomonas* organisms; they all produced N_2 and no NH_3. If cultures of the *Bacillus* and *Pseudomonas* organisms were sufficiently aerated there was no reduction of NO_3^-.

The ability to reduce nitrate has proved to be of value in the classification and identification of micro-organisms, e.g. in the yeasts, the genus *Hansenula* is differentiated from *Pichia* on the basis that only the former can grow on NO_3^- as a source of N. The gaseous products or nitrite formed by the activities of nitrate-reducing organisms are responsible for troublesome and unwelcomed consequences in certain industries. Thus NO_3^- and NO_2^- are commonly used for curing and preserving meat products and spoilation is often due to denitrifying bacteria. Some workers believe that denitrifying micro-organisms convert an appreciable amount of fertilizers such as $(NH_4)_2SO_4$ and KNO_3 into gaseous products and thus significantly decrease the amount of nitrogen available for plant growth [cf. 32]. After feeding on oat hay, which frequently contains large amounts of NO_3^-, sheep may show signs of methaemoglobinaemia (oat hay poisoning) and this condition is due to the absorption of NO_2^- formed by micro-organisms in the rumen [17]. If acidic conditions arise during the commercial production of alcohol by the fermentation of sugar molasses, large amounts of nitrogen peroxide are sometimes evolved. This is due to the acidic decomposition of nitrites which were formed from nitrates by micro-organisms during the processing of the molasses [32].

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CHAPTER IV

THE FIXATION OF NITROGEN

For several centuries it has been a matter of common observation and agricultural practice that soil impoverished by the growth of cereals can be revitalized by allowing the land to lie fallow or by growing leguminous plants, yet not until the end of the nineteenth century were adequate explanations forthcoming as to why these procedures caused such beneficial effects. They are in fact due to micro-organisms which, either themselves or when in association with leguminous plants, possess the ability to use the atmosphere as a source of nitrogen. The conversion of molecular nitrogen (N_2) into nitrogenous compounds which can be assimilated by the organisms concerned is termed *nitrogen fixation*. The ability to fix N_2 appears to be restricted to micro-organisms and even amongst them it occurs in but a few genera.

Priestley's claim that green plants absorbed N_2 as well as CO_2 and the suggestion of Sir Humphrey Davy that their nitrogen might be derived from the atmosphere by the agency of 'mushrooms and funguses' were the cause of much controversy and stimulated several investigations designed to test these statements by experiment [41]. Boussingault in 1838 was the first to show that, when they were grown in sand, the nitrogen content of clover plants increased yet that of wheat did not. Although in the years that followed, the swellings or nodules invariably found in the root systems of leguminous plants were frequently commented upon, half a century was to elapse before the classical experiments of Hellriegel and Wilfarth established that they were the site of the agency which enabled leguminous plants to fix atmospheric N_2. They found that nodules were formed only in non-sterile environments and that in contrast to cereals the growth of leguminous plants was normal even in the absence of fixed nitrogen (i.e. nitrate,

NH_4^+, etc.) provided they were able to develop these structures. By this time nodule formation was known to be the outcome of bacterial invasion of the root tissues [cf. 41, 35] and the organisms living in them were described by Frank under the name of *Bacterium radicicola* [18]. Contrary to the ideas of previous workers, Hellriegel and Wilfarth suggested that the bacteria were not parasites but lived in symbiotic association with the plant and endowed it with the ability to grow at the expense of atmospheric N_2 and thus be independent of an exogenous source of fixed nitrogen. Pure cultures of the nodular organisms, now placed in the genus *Rhizobium*, were first isolated by Beijerinck, who also found them free-living in the soil [3]. Like later workers, he was unable to demonstrate that these organisms fixed N_2 in the absence of the host plant, and the mechanism of N_2-fixation by the symbiotic system still awaits elucidation. All the strains of a given species of *Rhizobium* induce nodule formation in a restricted number of leguminous plants, termed a cross-inoculation group, and it is on this basis that the *Rhizobium* are classified into species, each species being specific for one cross-inoculation group [41, 1]. Although nodules may be formed, they are not always effective, i.e. capable of fixing nitrogen.

Isolation of free-living N_2-fixing organisms

During the latter half of the nineteenth century, Jodin and Berthelot provided evidence that certain free-living micro-organisms fixed atmospheric N_2, and pure cultures of bacteria exhibiting this property were eventually isolated by Winogradsky and Beijerinck. Each of these eminent bacteriologists used the enrichment culture technique with media which, apart from inorganic salts, contained only a substance such as glucose or mannitol as a source of carbon and energy: no nitrogenous compound was added. After being inoculated with soil the cultures were incubated in an atmosphere of air or nitrogen. Winogradsky thus isolated the anaerobe *Clostridium pasteurianum* which fermented glucose to acetic and butyric acids together with H_2 and CO_2 [49]. A few years later Beijerinck, using media

containing mannitol, isolated the two aerobes *Azotobacter chroococcum* and *Azotobacter agilis* (extremely motile) [4]. Apart from *Az. indicum*, *Azotobacter* spp. in general do not fix N_2 in an acidic environment, consequently their isolation is facilitated by the use of a neutral or slightly alkaline medium, *e.g.* one containing a buffer or $CaCO_3$. Furthermore, the incorporation of a small amount of sodium molybdate (5×10^{-6} per cent) is frequently advantageous since molybdenum appears to be of especial significance in organisms which fix N_2.

Organic compounds, other than those which serve as sources of carbon and energy, retard the growth of *Azotobacter* but not *Rhizobium*. It is, however, unlikely that the concentration of organic matter in the soil is ever great enough to affect the fixation of N_2 by *Azotobacter* in its natural habitat. Of the thirty-five compounds tested only aspartic acid, asparagine, glutamic acid, urea and adenine replaced molecular N_2 as a source of N for *Azotobacter* [20]. On the other hand, the growth of *Rhizobium* spp. was supported by any one of thirty-two organic nitrogen compounds and was luxuriant in rich organic media [30]. The optimal growth of fast-growing, but not slow-growing, strains of *Rhizobium* is dependent on an exogenous supply of a substance originally named coenzyme R and later identified as biotin [cf. 40].

Detection of N_2-fixation

Although from time to time the power to fix N_2 has been attributed to many other organisms, the majority of these claims must now be discounted on the grounds of faulty experimentation. It is very difficult, if not impossible, to eliminate all nitrogenous compounds from the materials used to make media, consequently growth in what is believed to be, apart from molecular N_2, a N-free medium is not a sufficient criterion for stating that an organism can fix nitrogen and, indeed, there is often no mention of control cultures incubated in the absence of molecular N_2. It is therefore relevant to consider the techniques used in the detection and quantitative study of N_2-fixation. Apart from

growth on a N-free media, these techniques involve gasometric analysis, nitrogen estimations by Kjeldahl procedures, manometry or the use of the nitrogen isotope N^{15}.

Gasometric analysis is used to detect whether there has been a decrease in the amount of gaseous N_2 in a closed system, whilst increases in fixed nitrogen can be determined by the use of a Kjeldahl technique. Although often used successfully [22], gasometric analysis is tedious and the elimination of experimental errors is not easy. The Kjeldahl method, which at first sight appears to be an ideal and technically simple procedure, has yielded many erroneous results, the reasons for which have been cogently assessed by Wilson [41]. The samples taken for analysis do not have a homogenous composition, and whilst a particular Kjeldahl procedure will estimate with precision the nitrogen content of one or more related compounds, it does not necessarily follow that the nitrogen in other compounds is estimated with a comparable degree of accuracy; e.g. the mere addition of water to dry seeds appeared to increase their nitrogen content as measured by the Kjeldahl procedure being used. None the less, provided such sources of error are borne in mind, the Kjeldahl technique has been, and is, of great value.

A more convenient, though indirect, method for studying N_2-fixation by free-living organisms is based on the use of the Warburg manometer, organisms being grown in the flask of the apparatus and the gas changes consequent on growth being followed in the usual manner. An obvious complication with aerobic organisms is that superimposed on any uptake of N_2, there is a concomitant uptake of O_2 due to respiration. Indeed, *Azotobacter* and *Rhizobium* possess the highest respiration rates known, the Q_{O_2} on glucose being of the order 1,000 to 2,000. Where large O_2 uptakes are expected, the usual Brodie manometric fluid is often replaced by one of greater density [e.g. Hg] in order that the manometer readings will be of convenient dimensions. If the rate of N_2-fixation is the factor limiting growth, then fixation by aerobic organisms can be followed in terms of an *increase* in the respiration rate, since the latter is directly

proportional to the mass of cytoplasm present in the system. With *Azotobacter*, the N_2 uptake is insignificant compared with the O_2 uptake, and is therefore neglected in calculating the results. Direct proof that such assumptions are justified was provided by Burk and Meyerhof, the originators of this technique [29, 7]. In their experimental conditions, irrespective of whether growth was followed by nephelometry, dry-weight measurements or total cell counts, the growth curves were all reasonably superimposable on the graph relating respiration rate with age of culture. Manometry has since been applied to the study of N_2-fixation by anaerobic bacteria, such as clostridia, *Desulphovibrio* and photosynthetic organisms [34].

Unequivocal evidence as to whether a system can fix N_2 is provided by the use of N_2 enriched with the non-radioactive isotope N^{15}, a technique first introduced by Burris [9] and which later proved to be of value in the elucidation of the route of N_2-fixation. The requisite control experiments showed that non-enzymic exchange reactions between molecular $N_2{}^{15}$ and compounds containing nitrogen were insignificant, and that *in vivo* there was no preferential uptake of one isotope over another, i.e. the *ratio* of the isotopes one to another in the gas-phase did not change throughout the course of the experiment. To detect N_2-fixation, the experimental material is placed in a closed system through which N_2 enriched with N^{15} is circulated by a Urey pump. The nitrogen-containing compounds of the experimental material may be fractionated prior to being converted into N_2 for analysis in the mass spectrograph. The technique is very sensitive and an increase of 0·01 atom per cent N^{15} above the normal distribution of 0·36 is regarded as being significant, and in certain conditions it is possible to detect the fixation of 0·001 μg. N [9, 10].

Organisms fixing N_2

In addition to *Cl. pasteurianum* and *Azotobacter* spp., there is now adequate evidence, in many cases based on experiments with N^{15}, that N_2-fixation is a property of several other species of clostridia [32], various photosynthetic

bacteria (species of *Rhodospirillum*, *Rhodopseudomonas*, *Rhodomicrobium*, *Chromatium* and *Chlorobacterium*) [19, 26 27, 28], *Desulphovibrio* [34], blue-green algae of the family Nostocaceae [15, 17, 43], and *Calothrix* [43]. Claims for fixation by yeasts and other fungi are as yet unsubstantiated. The successful demonstration that nodules detached from roots fix N_2 was dependent on the use of nodules from leguminous plants grown in the field rather than the greenhouse, performing the experiments immediately after detachment from the roots and subjecting only the soluble nitrogen compounds of the nodules to isotopic analysis [2]. The Leguminoseae is not the only family in which nodules are formed in consequence of microbial invasion, and the root nodules of the Alder (*Alnus*) and *Coriaria japonica* may also be concerned with the fixation of N_2.

Factors affecting N_2-fixation

There are no substantiated experiments in which N_2-fixation has been divorced from growth of the experimental material [24], consequently care is required in assessing whether the factor being studied directly affects the fixation mechanism: the observed effects may be no more than the result of interference with processes, such as the production of energy, which are essential to metabolism in general. For example, the influence of the partial pressure of oxygen (pO_2) on fixation by *Azotobacter* and by clover plants appears to be entirely explicable in terms of its effects on respiration, and in consequence, the availability of energy [7, 41]. Similarly, although molybdenum, iron, calcium and strontium have all been implicated in the fixation mechanism, it is difficult to decide whether this is their primary function. These difficulties cannot be circumvented by performing experiments in the presence of fixed nitrogen, since the latter induces a quicker rate of growth, and in all probability growth is then limited by different factors from those operative when the organisms are fixing molecular N_2. Whilst molybdenum undoubtedly influences the growth of *Azotobacter*, *Nostoc*, *Cl. pasteurianum* and leguminous plants, there is no direct evidence that it is

specifically concerned with the fixation mechanism (for discussion and references concerning trace element nutrition, see 8, 44, 21, 6).

Burk was the first to demonstrate that the rate of N_2-fixation by *Azotobacter* was a function of the partial pressure of N_2 (pN_2), the relationship between the two conforming to that expressed by the Michaelis-Menten equation for an enzymic reaction. The pN_2 at which fixation occurred at half the maximal rate (i.e. K_m) was 0·21 atm., an unexpectedly high value for a gaseous substrate [7]. In order to prepare gas mixtures containing different pN_2, Burk had used H_2 as a diluent gas since at that time H_2 was thought to be inert in biological systems. Later experiments, first with red clover plants [41, 47] and subsequently with *Azotobacter* [50], revealed that H_2 inhibited N_2-fixation and increasing the pH_2 caused a progressive decrease in the rate of growth: H_2 had no effect on growth in the presence of fixed nitrogen, e.g. $(NH_4)_2HPO_4$. Helium and argon exhibited no such inhibitory action, and in the absence of H_2, fixation by *Az. vinelandii* was maximal at a pN_2 of 0·15 atm. and half maximal at 0·02 atm. (Fig. 4.1). Hence, Wilson suggested that in natural conditions the pN_2 is not a factor limiting fixation. By comparing the rate of growth in air with that in gas phases containing, in addition to O_2, either 0·15 atm. N_2 or 0·3 atm. N_2, together with different amounts of H_2, Wyss and Wilson deduced that H_2 inhibited fixation in a competitive manner. Unlike *Azotobacter*, H_2 is a normal product in fermentations by *Cl. pasteurianum*, and although the pH_2 did not influence the rate of N_2-fixation by this organism it did affect the amount of N_2 fixed and the efficiency of the fixation process (i.e. mg. N fixed/g. glucose fermented) [32]. Wilson has noted that all the free-living organisms shown to fix N_2 also possess the enzyme hydrogenase [cf. 42]. He suggests that the latter may be involved in the fixation mechanism, since the hydrogenase activity of *Azotobacter* is greatly reduced, even in the presence of H_2, when growth is no longer dependent on the fixation of molecular N_2 [25]. Hydrogenase has not been detected in *Rhizobium* either free-living or symbiotic, or in

nodular tissues [45]. Nitrogen-fixation by *Azotobacter*, *Nostoc* and leguminous plants is inhibited in an apparently non-competitive manner by carbon monoxide in concentrations which cause little, if any, inhibition of respiration [48, 16]. Carbon monoxide is an isostere of N_2, and it may therefore replace N_2 on the surface of the enzyme concerned

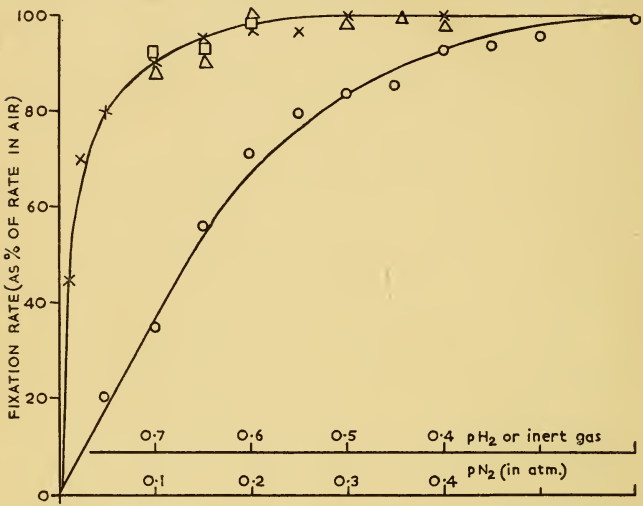

FIG. 4.1.—Nitrogen fixation by *Azotobacter* as a function of the partial pressure of nitrogen (pN_2) in the presence of different partial pressures of hydrogen (○), helium (×), or argon (△), or a partial vacuum (□): partial pressure of oxygen in all experiments, 0·2 atm. [50]

with the primary fixation reaction. Alternatively the fixation mechanism may involve a metal ion, perhaps combined in the prosthetic group of an enzyme, whose activity is lost on combination with CO. In this connection it may be of significance that the hydrogenase of *Az. vinelandii* is also inhibited by CO [44].

Role of ammonia in N_2-fixation

The idea has arisen that there is a key substance in

nitrogen-fixation, one which can be regarded both as the product of the fixation mechanism and as the substrate for the reactions by which inorganic nitrogen is incorporated into organic compounds. During the last decade, the elucidation of the nature of this substance has occupied the attention of two groups of workers, one associated with Virtanen in Finland [36] and the other with Burris and Wilson in the U.S.A. By using the isotope N^{15}, the American workers have obtained convincing evidence of the key role of NH_3 in the fixation of N_2, a concept first advanced by Winogradsky [49]. In the isotope experiments, the period of exposure to the substance enriched with N^{15} was not long enough for equilibrium to be established, and prior to isotopic analysis, the nitrogenous components of the experimental system were separated into fractions to facilitate location of the compounds with the highest concentration of N^{15}. For example, after acid hydrolysis, various amino-acids were isolated by classical precipitation procedures, or more recently, by the use of columns of ion exchange resins or starch [51, 43]. It was argued that if the assimilation of nitrogen involved a number of intermediates, then, before equilibrium was established, the concentration of the isotope would be the greater the nearer the intermediate to the substance initially enriched with N^{15}. Moreover, if a substance on the fixation pathway was supplied instead of, or in addition to, molecular N_2, it should be utilized not only as a source of nitrogen, but also in preference to its precursors (cf. simultaneous adaptation, p. 13) [11, 44].

The resultant distribution of the isotope amongst the constituents of *Az. vinelandii* was the same irrespective of whether nitrogen was derived from $N_2{}^{15}$ or $N^{15}H_4^+$, and of the cells' amino-acids, glutamic acid followed by aspartic acid, contained the highest concentration of N^{15} [12]. The establishment of such a distribution, even when the period of contact with $N^{15}H_4^+$ was short (3 min.), indicated that the enzymes responsible for the assimilation of NH_3 already existed in the bacteria. Moreover, the fact that NH_3 gave rise to the same distribution of N^{15} as N_2, implied that it was either itself on the pathway of N_2-fixation or at least

closely related to one of the natural intermediates. The high concentration of N^{15} in glutamic acid was indicative of its importance in the pathway of N_2-fixation. Such a conclusion was in keeping with the results of previous work with other organisms in which it had been established that glutamic acid occupies a key position in amino-acid metabolism and could be synthesized from NH_3 and α-ketoglutarate by the glutamic acid dehydrogenase system (cf. Chap. II).

Winogradsky was the first to note, during his experiments with *Cl. pasteurianum*, that ammonium salts inhibited N_2-fixation. *Azotobacter* responds in a similar fashion, both to ammonium salts and to compounds (urea, asparagine) which it can convert to NH_3 [46]. Since nitrate and nitrite were only inhibitory after a lag period, it was inferred that they were not utilized by *Azotobacter* until the appropriate enzymes had been formed by adaptation, and in fact there was no lag period with cells which had been grown in media containing NO_3^- and NO_2^-. Organic nitrogen compounds, e.g. aspartic and glutamic acids, were only moderately inhibitory, perhaps not unexpectedly since they are probably more concerned with intermediary metabolism rather than with the initial steps of the fixation mechanism.

Further isotope experiments provided additional evidence that the fixation of N_2 by *Cl. pasteurianum*, *Chromatium* and *Nostoc muscorum* is accomplished by essentially the same route as in *Az. vinelandii* [43]. In each of these organisms after exposure to $N_2{}^{15}$ or $N^{15}H_4$, the dicarboxylic amino-acids, and in particular glutamic, contained the highest concentration of N^{15}. Furthermore, the results of the experiments with *Cl. pasteurianum* [51] proved to be in some respects comparable with those of Virtanen with nodulated leguminous plants. Under certain conditions, the fixation of N_2 by *Cl. pasteurianum* and by the symbiotic system was accompanied by the excretion of nitrogenous compounds into the environment; the anaerobe excreted mainly NH_3 whilst the plant excreted aspartic acid, β-alanine and a small amount of an oxime, identified as oximinosuccinic acid. These substances are regarded as being products, not of catabolism, but of the processes directly concerned with

N_2-fixation. After exposing cultures of *Cl. pasteurianum* in the log phase of growth to N_2^{15}, the concentration of N^{15} in the NH_3 isolated from the medium was greater than that of the amide nitrogen of the cell protein which was in turn greater than the average level of the isotope in the proteins as a whole. This indicated that the excreted NH_3 was a product of the fixation processes and did not arise by deamination of amino-acids [51]. The excretion of NH_3 only occurs when the organisms are grown in certain media, and it is apparently an expression of a deficiency, probably of suitable organic acceptors, since supplementing the medium with biotin, *p*-aminobenzoic acid and α-ketoglutarate completely stopped the excretion of NH_3 and yet had little effect on the rate of N_2-fixation [43].

Role of hydroxylamine in N_2-fixation

Because leguminous plants apparently excreted only aspartic acid and substances related to it [38, 5], Virtanen originally concluded, especially in view of the presence of the oximino-compound, that hydroxylamine (NH_2OH) was the key product of the fixation mechanism. He proposed that NH_2OH condensed with oxaloacetate, a substance known to be present in nodulated roots, thus forming oximinosuccinic acid which was then reduced to aspartic acid. Many other workers were unable to repeat these experiments, and following a successful visit to Virtanen's laboratory, Wilson concluded that excretion was observed only when the rate of photosynthesis was not sufficient to supply enough materials for the utilization of all the products of the fixation mechanism [41]. The later discovery [39] that glutamic acid was present amongst the excreted substances implied that many of the results of the leguminous plant experiments could also be interpreted in favour of the importance of NH_3 in the fixation mechanism. Whilst it seems most probable that nitrogen enters into organic combination in the form of NH_3 and not NH_2OH, the possibility that NH_2OH is a precursor of the NH_3, and perhaps, in certain circumstances, reacts directly with an organic acceptor, has not yet been excluded [cf. 37].

A series of well-controlled experiments has failed to find any evidence for the participation of NH_2OH in the fixation of N_2 by *Azotobacter* and *Cl. pasteurianum* [31, 33]. Hydroxylamine in concentrations greater than about 2 μg./ml. was toxic, and growth, when it occurred, could be accounted for in terms of NH_3 produced by the spontaneous decomposition of NH_2OH. Even with $N^{15}H_2OH$ the results were difficult to interpret because the amount of nitrogen involved was too small to permit significant determinations. The oximes of α-ketoglutarate, oxaloacetate and pyruvate were slowly utilized by *Cl. pasteurianum* but not by *Azotobacter* [34].

Pathways of N_2-fixation

There has been little support in recent years for the fixation of nitrogen being explained in terms of an oxidative pathway involving compounds such as nitrous oxide and hyponitrous acid. There is now a considerable amount of evidence that it is probably a process involving a number of reductive mechanisms which terminate in the formation of NH_3. Whilst the steps between molecular N_2 and NH_3 are completely unknown some hypothetical pathways are shown in Fig. 4.2 (details of other schemes will be found in reference 44). Although the fixation of N_2 by cell-free systems has yet to be confirmed, past failures [24] may be attributed, at least in part, to the harsh procedures employed in the preparation of the extracts.

In spite of their diverse nature, bacteria, blue-green algae and leguminous plants all apparently fix nitrogen by mechanisms which, if not identical, have at least many features in common [43]. They all respond in a similar manner to changes in the partial pressure of N_2, H_2 and CO and their K_m values for N_2 are of the same order. In view of the repeated failures to demonstrate N_2-fixation by free-living cultures of *Rhizobium*, interest with regard to the symbiotic system is centred around the question why association with the leguminous plants should endow one or other of the symbionts with this ability. A noticeable feature of the association is the presence of a red haemo-

globin-like pigment ('leghaemoglobin') in the effective nodules [44]. The pigment is only formed after the nodules have been established and concurrent with the change to a green colour, their ability to fix N_2 declines. The pO_2 in the nodules is relatively low and the pigment may serve to store and transport O_2 to the *Rhizobia*, which, it will be remem-

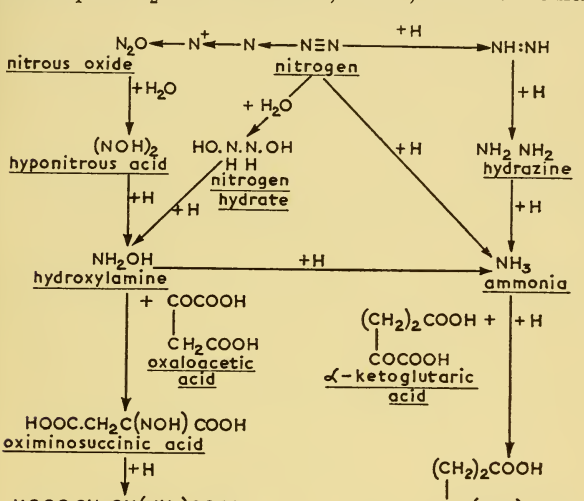

FIG. 4.2.—Hypothetical pathways for the fixation of molecular nitrogen

bered, possess a high rate of respiration. Some workers have suggested that the haemoglobin participates directly in the fixation mechanism. For example, NH_2OH is decomposed by haemoglobin (Hb) *in vitro*, thus [14]:

$$NH_2OH + 2Hb^{++} + H_2O \longrightarrow 2MetHb^{+++} + NH_3 + 2OH^-$$

$$2NH_2OH + 2MetHb^{+++} \longrightarrow 2Hb^{++} + 2H_2O + N_2 + 2H^+$$

and it is possible that the fixation of nitrogen is accomplished by the reverse of these reactions. However, claims for the natural occurrence of methaemoglobin in the nodules

have been seriously challenged [23]. The addition of haemoglobin increases the respiration of *Rhizobium*, but this appears to be an indirect effect [13] and not connected with the transport of O_2.

It will be evident that there are still many aspects of nitrogen fixation to be explored, and apart from their biochemical interest, their economic importance should not be underestimated. The growth of plants, the leaching effects of rain-water and the activities of denitrifying organisms all tend to remove from the soil the nitrogenous compounds which are essential to the continued existence of most forms of plant life. This loss is in part restored naturally by the decomposition of plants and animals, and artificially by the application of inorganic or organic fertilizers. Fixed nitrogen compounds produced commercially probably account for no more than 15% of the nitrogen returned annually to the soil. By far the greatest proportion is due to N_2-fixation by biological agents, and it has been estimated that symbiotic systems and free-living organisms are responsible respectively for returning to the soil $5{\cdot}46\times10^6$ and $4{\cdot}37\times10^6$ tons of nitrogen per year, yet, even allowing for this, there appears to be an annual overall loss in soil nitrogen [42].

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CHAPTER V

SYNTHESIS OF AMINO-ACIDS

From the vast amount of information now available it is clear that glutamic acid and aspartic acid occupy a key position in amino-acid metabolism, and, of the two, the former is the more important. Glutamic acid can be synthesized from NH_3 and α-ketoglutaric acid by the glutamic dehydrogenase system (p. 12) and aspartic acid from NH_3 and fumaric acid by aspartase (p. 23). The direct addition of an inorganic compound of nitrogen to the appropriate carbon skeleton does not appear to be a general route for the synthesis of amino-acids, and the importance of the dicarboxylic amino-acids is in part due to the fact that they contain nitrogen in a form which can be transferred to suitable acceptors and thus utilized for the synthesis of other nitrogenous compounds, e.g. the conversion of citrulline to arginine (p. 70) and the synthesis of amino-acids by transamination. Moreover, it will become clear from the following paragraphs that several amino-acids may be derived from other preformed amino-acids—for example, proline and ornithine can be synthesized from glutamic acid (pp. 69, 70). Many organisms use an inorganic form of nitrogen, such as molecular N_2, NH_3 and NO_3^-, as a source of this element, and it is now generally held that the first steps in the utilization of molecular N_2 and nitrate for this purpose involves their conversion to NH_3 (see Chaps. IV and III). It is therefore worthy of note that the glutamic dehydrogenase system and aspartase are mechanisms which enable inorganic nitrogen in the form of NH_3 to be incorporated into an organic molecule.

Transamination and amino-acid synthesis

A transaminase catalyses the reversible transfer of the amino group of one amino-acid to the α-keto acid corresponding to another amino-acid:

$$R^1CH(NH_2)COOH + R^2COCOOH \rightleftharpoons R^1COCOOH + R^2CH(NH_2)COOH \quad \text{(i)}$$

Reactions of this type were first discovered in animal tissues by Braunstein and Kritzmann, who concluded that the amino groups of several amino-acids could be transferred in such a manner provided one of the participants in the system was a dicarboxylic acid, i.e. aspartic, glutamic, oxaloacetic, or α-ketoglutaric acid [4]. Cell-free preparations of what were believed to be two distinct transaminases were obtained, one catalysed reactions ii and iii, and the other reaction iv:

$$\text{glutamate} + \text{pyruvate} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{alanine} \quad \text{(ii)}$$

$$\text{glutamate} + \text{oxaloacetate} \rightleftharpoons \alpha\text{-ketoglutarate} + \text{aspartate} \quad \text{(iii)}$$

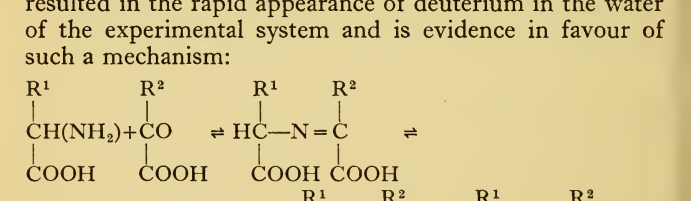
$$\text{aspartate} + \text{pyruvate} \rightleftharpoons \text{oxaloacetate} + \text{alanine} \quad \text{(iv)}$$

Later workers isolated two enzyme systems, specific for reactions ii and iii respectively, and Kritzmann's second transaminase was shown to be an artifact and due to a mixture of these two enzymes with catalytic amounts of glutamate functioning as a carrier between the two systems [23].

Similar transaminase systems were later found in bacteria, yeasts [39*a*] and *Neurospora* [17*a*, *b*]. Washed cell suspensions of various bacteria (staphylococci, streptococci, pneumococci, enterobacteria, *Az. agilis*, and *Ps. pyocyanea*) catalysed the transfer of the amino group of glutamic acid to oxaloacetic acid (reaction iii), and cell-free preparations of *Strep. faecalis* accomplished both reactions ii and iii, the equilibrium being in favour of the synthesis of alanine and aspartic acid respectively [34, 35]. Like the animal transaminases, the bacterial enzymes possess a prosthetic group of pyridoxal phosphate. The glutamic-aspartic transaminase of *Strep. faecalis* had been partially resolved and activity was restored by the addition of synthetic pyridoxal phosphate or natural codecarboxylase. The advent of paper chromatography has facilitated the detection and identification of small quantities of amino-acids and has been used to demonstrate that several amino-acids can transfer their amino groups to α-ketoglutarate. These experiments have been performed with *Esch. coli*, *Ps. fluorescens*, *B. subtilis* [14]

and *Lb. arabinosus* [36*a*] and also with animal tissues [26]. Unequivocal proof that such results are due to transamination awaits the isolation of the appropriate enzymes, and, indeed, a glutamic-tyrosine transaminase and a glutamic-phenylalanine transaminase have been isolated from *Esch. coli* and shown to contain prosthetic groups of pyridoxal phosphate [14]. All known transaminases are specific for the L-isomers of the amino-acids.

Our conceptions of the mechanism operative in biological transamination are based on analogy with *in vitro* systems. Transamination is a true transfer process and there is no evidence at all that the amino group becomes free as NH_3 at any stage in the reaction. Herbst has proposed that a type of Schiff's base is formed by condensation between the amino and keto groups of the two substrates and that this is followed by a molecular rearrangement involving the alpha hydrogen atom of the amino donor, after which the base is ruptured by hydrolysis [25]. Incubation of alanine containing deuterium in the alpha position with α-keto-glutarate and a mammalian glutamic-alanine transaminase resulted in the rapid appearance of deuterium in the water of the experimental system and is evidence in favour of such a mechanism:



It is to be remembered that pyridoxal phosphate itself contains a carbonyl group and Snell, on the basis of non-enzymic chemical experiments, has made the following proposal [42]:

amino-acid A+pyridoxal phosphate ⇌
keto acid A+pyridoxamine phosphate
pyridoxamine phosphate+keto acid B ⇌
amino-acid B+pyridoxal phosphate

Synthetic pyridoxamine phosphate activated some preparations of partially resolved bacterial transaminases [48], and though first reported not to restore activity to a resolved preparation of the pig heart glutamic-aspartic transaminase, later workers showed that it was as active as pyridoxal phosphate provided it was incubated with the apoenzyme for 30–60 minutes before adding the substrates [36*b*]. Gunsalus and Tonzetich have recently demonstrated that preparations of *Esch. coli* catalysed transamination reactions between pyridoxal and glutamic acid and between pyridoxamine and α-ketoglutarate. Hence, while there is some reason to believe that the prosthetic group of the transaminases can function as a carrier of amino groups, direct proof that it does so has still to be obtained.

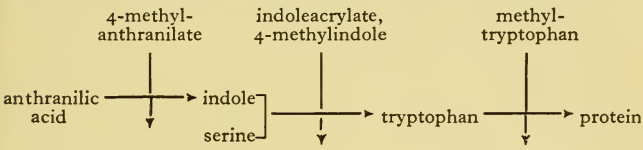
Owing to the lack of essential data, it is not yet possible to assess whether transamination is of key importance in amino-acid synthesis. The available information, admittedly extremely incomplete, indicates that the transaminases are widely distributed in micro-organisms, at least in those non-exacting to amino-acids. Whether or not there is a complete series of enzymes capable of reacting with the keto acids corresponding to all the natural amino-acids is still not known. If transamination is involved in the synthesis of an amino-acid, then it follows that the organism must be able to synthesize the appropriate keto acid. Except for pyruvate, oxaloacetate and α-ketoglutarate, virtually nothing is known about the synthesis of these compounds, and although the two former acids are known to be produced in the intermediary metabolism of many organisms, conclusive evidence of the ability to synthesize α-ketoglutarate is only available for *Ps. fluorescens*, *Az. agilis* and *Sac. cerevisiae*. Although in recent years it has been thought that glutamic acid was the only amino-acid able to transfer an amino group to a wide variety of keto acids, there is now some evidence that aspartic acid can transaminate with acids other than α-ketoglutarate, e.g. there is an aromatic amino-acid—aspartic acid transaminase in *Esch. coli* [39*b*]. Furthermore, transamination reactions are now known in which dicarboxylic acids do not participate, e.g. *Brucella abortus* appears to

possess a leucine-alanine transaminase [1*c*]. Transamination can therefore be visualized as being of some significance in amino-acid synthesis provided the organism can either synthesize one or more amino-acids from ammonia by a direct route (e.g. the glutamic acid dehydrogenase system or aspartase), or obtain suitable amino group donor amino-acids from the environment.

Metabolite analogues and the elucidation of metabolic pathways

Many enzyme systems are inhibited in a competitive manner by substances similar in chemical structure to their normal substrates: such substances are known as metabolite analogues and have been used, for example, to study the synthesis of tryptophan by *Salmonella typhosa* [15]. Although a reaction inhibited in this fashion may be essential for growth, nevertheless, growth may be possible provided the medium is fortified in some way, i.e. by the addition of substances antagonizing the effects of the inhibitor. Ideally, the substrates of the inhibited reaction act as competitive antagonists, whereas the products act in a non-competitive manner. The growth of freshly isolated strains of *Salm. typhosa* is dependent on tryptophan or indole, but non-exacting strains frequently arise. The inhibitory effects of β-indoleacrylic acid on the growth of a non-exacting strain were completely overcome by the addition of tryptophan. Indole accumulated in the media of non-exacting strains growing in the presence of limited amounts of tryptophan and sub-lethal amounts of indoleacrylic acid, and was presumed to be the substrate of the reaction blocked by the inhibitor. Serine was a powerful antagonist and high concentrations of this amino-acid also decreased the accumulation of indole. Fildes therefore concluded that tryptophan was synthesized by the condensation of serine with indole and that indoleacrylic acid inhibited this reaction. The work of Snell and Schweigert had already indicated that anthranilic acid might be an intermediate in the synthesis of tryptophan by *Lb. casei* and *Lb. arabinosus*, and it was later found that irrespective of their tryptophan requirements, strains of *Salm. typhosa* secreted anthranilic acid into the

culture medium [40]. Since the amount of anthranilic acid produced by an exacting strain was greater than the amount of indole utilized, it was unlikely that the former was derived from the latter. The inhibitory effect of 4- or 5-methylanthranilic acid on the growth of the non-exacting strain was reversed by anthranilic acid, indole or tryptophan [16]. These results are summarized in the following scheme, vertical arrows denoting the site of action of inhibitors:



Another example of antagonism in amino-acid metabolism comes from studies of the nutrition of *Bacillus anthracis* and serves to emphasize that growth may be dependent not only on the presence of certain amino-acids but also on their relative concentrations [19]. Although *B. anthracis* would not grow on a synthetic and complete amino-acid medium from which valine, leucine or isoleucine had been removed, growth did occur in the absence of all three of these amino-acids. When valine was present, no growth was possible unless a suitable amount of leucine had also been added, whilst growth in the presence of isoleucine was conditional on the addition of both valine and leucine. Since these amino-acids are of comparable chemical structure, Gladstone concluded that they are synthesized by similar, if not identical, routes and that the addition of only one of the acids resulted in the competitive inhibition of one or more of these routes. Several examples of this type of effect have been found during experiments with *Neurospora* mutants [cf. 45].

Mutants

Much valuable information concerning the routes of biological synthesis of natural compounds has been derived by the use of mutants, i.e. from organisms genetically different from the parent strains. If this difference results in inability

to synthesize a compound essential for life, the mutant is unable to grow unless it can obtain from the environment at least one of the products of the reaction which it is unable to accomplish. Such a reaction is often referred to colloquially as a 'genetically blocked reaction'. Beadle and Tatum predicted that it should be possible to deduce the sequence of reactions in biosyntheses from the range of compounds which replace the substances required by nutritionally-exacting mutants. The organisms used in these studies include *Penicillium notatum*, *Aspergillus niger*, *Asp. nidulans*, *Ophiostoma*, *Esch. coli* and *B. subtilis*, but the ascomycetes *Neurospora crassa* and *N. sitophila* still remain the most suitable if precise genetic data is also required. Most natural (i.e. 'wild type') strains of *Neurospora* grow on a simple medium containing mineral salts, biotin, an inorganic source of N and an organic source of C and energy (e.g. sucrose, sorbitol) and must therefore possess the wide variety of enzymes required for the synthesis of all the normal constituents of cytoplasm. The vegetative phase reproduces asexually by conidia and micro-conidia; sexual reproduction is only possible between gametes from parents of opposite mating types. Much is known concerning the genetics of *Neurospora* [see 5] and since the vegetative phase is haploid, there are in contrast with diploid organisms, no problems concerning the dominance of one character over another. Strains whose nutritional requirements are different from the parent type may arise naturally by spontaneous mutation, but such mutations are often few in number and natural selection does not favour their survival. In order to increase the chance of isolating such mutants, the mutation rate is artificially increased by exposing the conidia to ultra-violet light, X-rays or chemical mutagens, e.g. mustard gas. The conidia are then transferred to the protoperithecia of wild type *Neurospora* of opposite mating type, and in consequence asci develop, each ascus containing eight spores. One spore from each ascus is transferred to a solid medium containing the minimal requirements for growth (minimal medium), and after incubation the colonies that have developed will be of the wild type. Their position is noted

and agar containing known additional nutrients is layered over the original plate. Any new colonies which develop after further incubation are derived from mutant spores, and after being subcultured their nutrition can be studied in more detail. Special techniques may be required in order to promote the formation of discrete colonies, particularly with organisms whose growth tends to spread (many strains of *Neurospora*). A more laborious method of isolation is to subculture each ascospore on a rich medium, i.e. one containing amino-acids and growth factors, and then transfer to a minimal medium. If there is no growth on the latter, the nutrition of the parent colony is then examined further. Other more efficient, technically easy and less laborious methods for selecting mutants of various organisms have been described [18, 31, 32].

Using such techniques, a number of mutants have been obtained which are exacting towards a particular amino-acid, growth factor, purine or pyrimidine. Many appear to be unable to perform reactions expected to take place in one step, e.g. the amination of inosine to form adenine, and Beadle and Tatum have advanced the hypothesis that each enzyme is controlled by a specific gene, and any change in the latter is reflected by an alteration in the enzyme's activity. Although the evidence is indirect and has been criticized by Delbruck [11] an analysis of the available information shows that at least 73% of the genes of *Neurospora* have only one function, and there are no indisputable examples of genes with two or more functions [30]. It must be stressed that mutation may involve modification rather than complete loss of the gene and the corresponding enzyme, e.g. a *Neurospora* mutant unable to synthesize tryptophan from indole and serine still possessed the requisite condensing enzyme, though in an inactive state [22]. (Cf. also the synthesis of pantothenic acid [51]).

In order to show whether mutants requiring the same factor are due to mutation of either the same gene or completely different genes, one of two tests may be applied. In organisms with a sexual cycle (e.g. *Neurospora*), each mutant is mated with a parent of known genetic composition and the

percentage recombination of characters obtained in the progeny of each cross will indicate whether or not the mutants are the outcome of mutation at the same gene locus. The cells of fungi are multinucleate and it frequently happens that hyphae from two parents will fuse and thus form a structure known as a heterocaryon which contains nuclei derived from each of the parent hyphae. Consequently a heterocaryon formed between two genetically identical mutants will have the same growth requirements as the two parent mutants. But the heterocaryon from genetically different mutants will grow in the complete absence of such compounds since the metabolic deficiencies of the nuclei derived from one parent will be complemented by the activities of the nuclei from the other parent.

Primarily because of the ease with which they can be isolated, mutants of penicillin-sensitive bacteria have been used in many recent investigations [9]. Mutation is brought about by irradiation with ultraviolet light in doses sufficient to kill 99·9% of the organisms and then the suspension is cultured in a rich medium, washed and transferred to minimal medium containing penicillin. The non-mutants grow and are consequently killed by the penicillin. The suspension is then plated on to a rich medium and the colonies appearing are usually those of nutritionally exacting mutants.

Having isolated a number of mutants exacting towards a particular substance, other substances likely to be intermediates in its synthesis are then tested for their ability to promote growth, since theoretically the product of the blocked reaction or any compound derived from it should be active in this respect, provided the organism is permeable to such substances. The sequence of intermediates in a biosynthesis may then be deduced by arranging the mutants in order according to the range of compounds supporting growth. It is to be expected that the further the blocked reaction from the end-product, the greater the number of compounds utilized by the mutant. For example, in the synthesis of D from A by:

$$A \xrightarrow{a} B \xrightarrow{b} C \xrightarrow{c} D$$

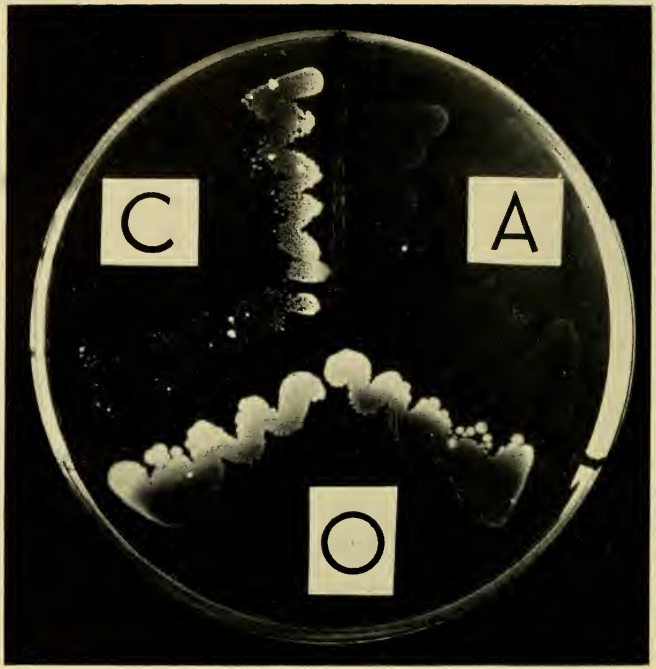


PLATE 1.—Syntrophism among arginine requiring mutants of *Esch. coli*. Mutant O responds to ornithine, citrulline or arginine; C to citrulline or arginine; A to arginine only. Photograph of growth after 48 hr. at 37° C. on a medium containing suboptimal amounts of required nutrients. Note the enhanced growth of C and O due to the secretion by A of a substance (citrulline?) which can be utilized by C and O. Similarly the enhanced growth of O due to the secretion by C of a substance (ornithine?) utilized by O [7]

the growth of a mutant incapable of reaction *a* will be supported by *B*, *C* or *D*; if incapable of *b*, by *C* or *D*; and if incapable of *c*, the mutant will only grow in the presence of *D*.

For convenience, mutants are described either by adding the suffix '-less' to the substance required for growth, e.g. argininеless denotes exacting towards arginine, or by using the term auxotroph, e.g. an arginine auxotroph.

Growth in the presence of suboptimal amounts of the product of a blocked reaction may result in the excretion of the precursor of this reaction into the medium. Consequently when two related mutants are streaked near one another on a solid medium containing suboptimal amounts of required

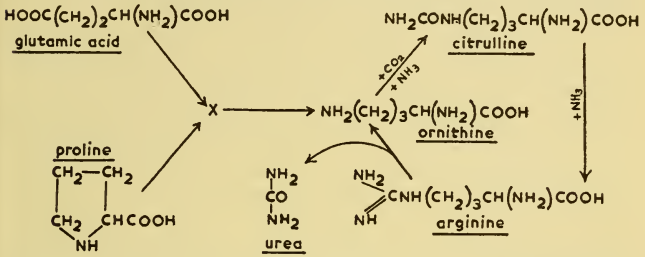


FIG. 5.1.—The arginine cycle and the route of arginine synthesis in *Neurospora crassa* and *Penicillium notatum*

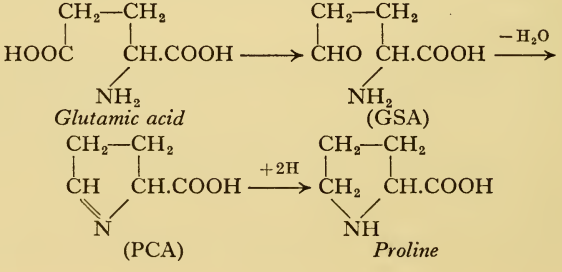
nutrients, one may secrete a substance which is used directly by the other or changed by it into a form which can be utilized by the former mutant. The enhanced growth which results is readily visible and this phenomenon of 'syntrophism' has been widely exploited by Davis [7] (Plate I).

Arginine synthesis

Seven genetically distinct argininеless mutants of *N. crassa* were isolated and of these, four grew on ornithine, citrulline or arginine; two on citrulline or arginine and one on arginine only. The organism also possessed arginase and urease and it was concluded that there is an 'arginine cycle' in *Neurospora* (Fig. 5.1) comparable to that described by Krebs in mammalian liver. It can be deduced from the genetic data that there are at least four steps in the synthesis of ornithine

and two in the conversion of ornithine to citrulline. In liver, the synthesis of citrulline from ornithine, CO_2 and NH_3 proceeds by a mechanism utilizing metabolic energy and with carbamylglutamic acid as an essential co-factor. Contrary to expectation, the latter does not function by transferring the carbamyl group directly to ornithine. In the presence of ATP and Mg^{++}, the citrulline combines with aspartic acid and the product subsequently undergoes hydrolysis to yield arginine and malic acid [38]. Comparable systems have not yet been described in micro-organisms. In *Lb. arabinosus* glutamine appears to play an essential role in arginine synthesis, the amide group being used in the formation of citrulline from ornithine [37*b*].

A mutant of *P. notatum* grew on arginine, citrulline, ornithine or proline, whilst another grew on either of these amino-acids or glutamic acid. A third was known to grow only on proline, indicating that proline is not itself on the direct route of arginine synthesis but is probably related to a precursor of ornithine [3, also cf. 43]. The possible relationships between glutamic acid, proline and the arginine cycle are shown in Fig. 5.1. Studies of the nutrition of naturally occurring strains of lactobacilli [50] and mutants of *Esch. coli* provided evidence that the mechanism of arginine synthesis in bacteria is the same as in the fungi. Using mutants of *Esch. coli*, Davis and his colleagues [8] have shown that proline is formed by the reduction of Δ^1-pyrroline-5-carboxylic acid (PCA), a compound formed from the γ-semialdehyde of glutamic acid (GSA).



The relationships of these compounds to ornithine has still to be fully elucidated [17*b*]. The conversion of glutamic acid to ornithine probably proceeds via N-acetylglutamic acid → N-acetylglutamic acid γ-semialdehyde → α-N-acetylornithine → ornithine [see 1*a*].

Tryptophan and nicotinic acid

The interrelationships between tryptophan, the other aromatic amino-acids and nicotinic acid, as revealed by experiments with *N. crassa* and *Esch. coli*, are summarized in Fig. 5.2. Initially, two tryptophanless mutants of *Neurospora* (10575 and 40008) were isolated, both utilized indole in place of tryptophan but only one (40008) utilized anthranilic

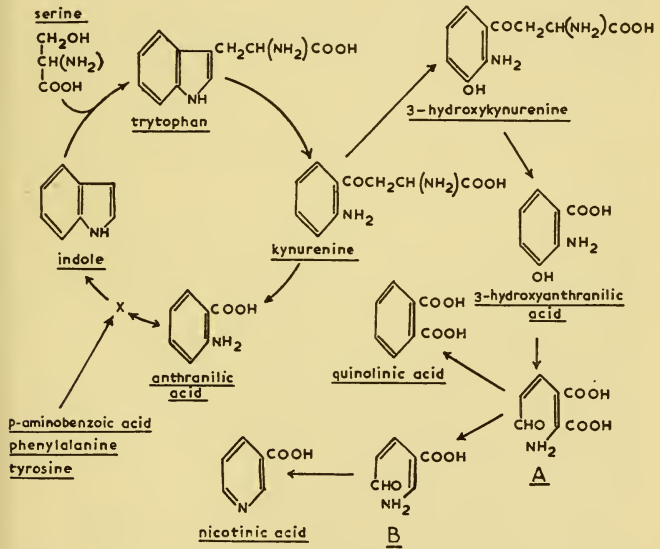


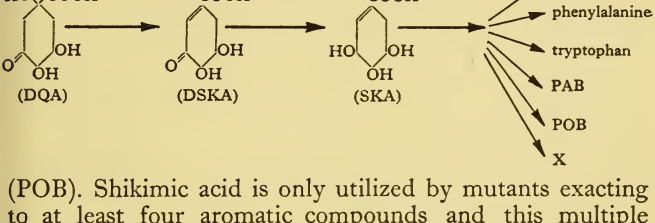
FIG. 5.2.—The tryptophan cycle and the synthesis of nicotinic acid in *Neurospora crassa*. Compounds A and B are hypothetical intermediates, and the details of the relationship between tryptophan and the other aromatic amino-acids and PAB are not yet known (see pp. 72–3) [24]

acid. Growth of 10575 in the presence of limiting amounts of tryptophan resulted in the appearance in the medium of a substance supporting the growth of 40008. This material, presumably the substrate of the blocked reaction, was later isolated and identified as anthranilic acid. The reaction sequence is therefore anthranilic acid → indole → tryptophan. The rate of uptake of indole by 10575 was found to be a function of the concentration of L-serine in the medium, and after growth had ceased tryptophan was excreted into the medium. The enzyme forming tryptophan by the condensation of serine with indole was studied in cell-free homogenates of *Neurospora* mycelia and shown to contain a prosthetic group of pyridoxal phosphate [49]. The mechanism by which anthranilic acid is converted into indole remains unknown. By using isotopes it has been shown that the carboxyl group of the former does not give rise to any of the carbon in the indole nucleus of tryptophan [37*a*].

A detailed investigation of *Neurospora* mutants able to grow on tryptophan or nicotinic acid has confirmed the conclusion drawn from animal nutrition experiments that the metabolism of these two compounds is interrelated. Furthermore, Haskins and Mitchell have proposed that, at least in *Neurospora*, there is a 'tryptophan cycle' [24]. By using the appropriate mutants and growth conditions, 3-hydroxyanthranilic acid, kynurenine and quinolinic acid have all been isolated from culture filtrates. Like *Lb. arabinosus*, the growth of some, but not all, nicotinic acid auxotrophs of *Neurospora* is supported by quinolinic acid, although only in high concentrations [21]. It is therefore possible that quinolinic acid is a by-product derived from the substrate of a blocked reaction, rather than a direct intermediate in the synthesis of nicotinic acid.

Certain mutants of *N. crassa* and *Esch. coli* require tryptophan, phenylalanine, tyrosine and *p*-aminobenzoic acid (PAB), all in large amounts, before there is even slow growth, indicating that these four compounds may be derived from a common precursor and that the synthesis of anthranilic acid and tryptophan is connected with the

metabolism of the other aromatic amino-acids. As a result of a suggestion made by Stanier, Davis found that this quadruple requirement could be replaced by shikimic acid, an alicyclic compound known to occur in plants. This acid has now been isolated from the culture filtrate of a mutant of *Esch. coli* and unequivocally characterized. A precursor of shikimic acid (SKA) has recently been isolated and identified as 5-dehydroshikimic acid (DSKA) which is in turn probably derived from 5-dehydroquinic acid (DQA) [8]. Since growth in the presence of the four aromatic acids was slow and became maximal on the addition of shikimic acid or filtrates from wild-type cultures, Davis deduced that the mutants required at least one other aromatic substance, and one of these has been identified as *p*-hydroxybenzoic acid



(POB). Shikimic acid is only utilized by mutants exacting to at least four aromatic compounds and this multiple requirement is probably due to the mutation of a single gene. It is not yet possible to state whether shikimic acid is in fact a simple precursor of all these aromatic nitrogen compounds. Davis has suggested [7, 10] that the apparent complexity in growth requirements is the result of interference with the synthesis of a key substance which is responsible for the integration of various parallel and related pathways of biosynthesis (cf. valine-isoleucine, pp. 65, 76). Unlike mammals, *Neurospora* and *Esch. coli* cannot convert phenylalanine to tyrosine.

Cysteine and methionine

Sulphur is found in organic combination in the amino-acids cysteine and methionine, and most organisms can utilize inorganic forms of sulphur at any oxidation level as

a complete source of this element. Mutants exacting towards various sulphur compounds are the easiest to produce and isolate. Of four methionineless mutants of *N. crassa*, only one specifically required methionine, homocysteine was just as effective for two of the mutants, whilst the other grew on cysteine, homocysteine or methionine. The culture filtrate

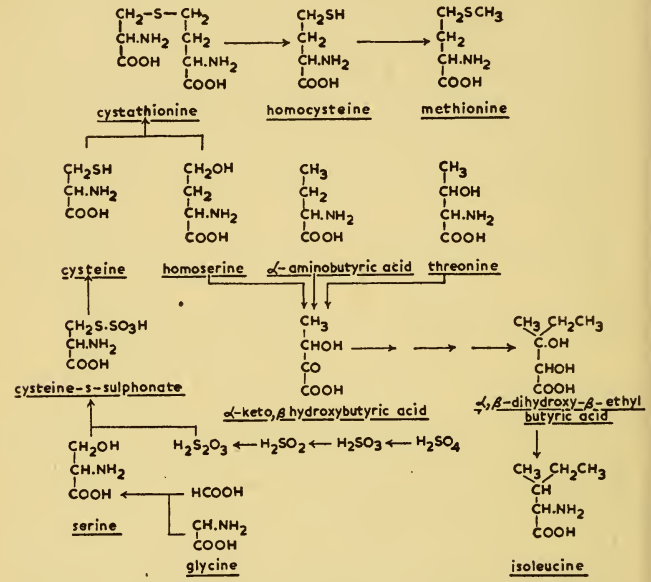


FIG. 5.3.—Pathways for the synthesis of cysteine, methionine and isoleucine

of one homocysteine auxotroph was found to contain a substance capable of supporting the growth of the other homocysteine autotroph and also of the mutant which would grow on cysteine. This substance was isolated and identified as cystathionine [29], and the suggested biosynthetic sequence is shown in Fig. 5.3. Methionine is probably synthesized by methylation of the homocysteine produced by the cleavage of cystathionine (see p. 149).

A *Neurospora* mutant requiring threonine grew poorly unless the medium was also supplemented with methionine, cystathionine or homocysteine, indicating that all these compounds were derived from a common precursor. The latter is probably homoserine since this amino-acid can replace both threonine and the sulphur-containing amino-acids [46]. Moreover, if homoserine can couple with cysteine the product would be cystathionine. As regards the incorporation of inorganic forms of sulphur into organic compounds, it has been suggested that this proceeds via the synthesis of cysteine by cysteine desulphurase, an enzyme known to decompose cysteine into H_2S, NH_3 and pyruvic acid. There is, however, no evidence that the enzyme can catalyse the reverse reaction. In *Asp. niger*, *Asp. nidulans* and *P. notatum* [27], thiosulphate, and not sulphide, may be an important intermediate in the synthesis of cysteine. The sulphur requirements of thiosulphate auxotrophs of *Asp. nidulans* were satisfied by cysteine-S-sulphonate (a thiosulphate derivative of serine) and growth was extremely luxurious in the presence of serine and thiosulphate [28]. Hockenhull therefore proposed that the route of sulphur utilization involves reduction of sulphate to sulphite which is then converted to thiosulphate, perhaps with the intermediate formation of sulphoxylate, and that finally thiosulphate is condensed with serine or some other C_3-compound. A different sequence has been proposed for *Ophiostoma multiannulatum* [see 5] and *N. crassa*:

C_3-compound (alanine?) plus SO_4^{--} $\longrightarrow$ cysteic acid $\longrightarrow$ cysteine sulphinic acid $\longrightarrow$ cysteine

Lysine and threonine

The route of lysine synthesis in *Neurospora* appears to be different from that in *Esch. coli*. Lysine auxotrophs of *Neurospora* were able to utilize α-aminoadipic or ε-hydroxy-α-aminocaproic acid but not α-ketoadipic, α,α′-diaminoadipic or α,ε-diaminopimelic acid. The biosynthetic sequence in *N. crassa* is believed to be:

α-aminoadipic acid $\longrightarrow$ ε-hydroxy-α-aminocaproic acid $\longrightarrow \longrightarrow \longrightarrow$ lysine [20]

Unlike *Neurospora*, *Esch. coli* contains α,ε-diaminopimelic acid (DAP) and possesses a specific enzyme converting it into lysine and CO_2 (p. 29). This decarboxylase is not present in those lysineless mutants which accumulate large amounts of DAP in their culture media. The lysine requirements of other mutants were satisfied by DAP but not by α-aminoadipic or α-amino-ε-hydroxycaproic acid, and the conclusion was reached that DAP is the immediate precursor of lysine in *Esch. coli*. Moreover, DAP and threonine may be derived from a common precursor, and α-aminobutyric acid may be an intermediate in the synthesis of threonine from homoserine (Fig. 5.3) [9].

Valine, isoleucine and threonine

A mutant (16117) of *N. crassa* would only grow when provided with both L-valine and L-isoleucine, yet as far as could be ascertained it differed in only one gene from the wild type parent. For optimal growth, the ratio of L-valine to L-isoleucine was critical (7:3) and increasing the concentration of either acid adversely affected growth. The α-keto acids corresponding to valine and isoleucine ('ketovaline' and 'ketoisoleucine') supported the growth of other mutants, and 16117 would grow in the presence of isoleucine and 'ketovaline' but not valine and 'ketoisoleucine'. Moreover, 'ketoisoleucine' inhibited the growth of another mutant requiring only 'ketovaline'. Bonner has suggested that 16117 is unable to synthesize isoleucine and that the substrate of the blocked reaction ('ketoisoleucine'?) accumulates and competitively inhibits the synthesis of valine (from 'ketovaline'?), with the result that the mutation of one gene appears to bring about the blocking of two reactions [3]. After 16117 has grown in the presence of isoleucine and valine, the medium contains α,β-dihydroxy-β-ethylbutyric acid, a substance replacing isoleucine for an auxotroph of *Esch. coli*; no 'ketoisoleucine' could be detected [1*b*]. Isoleucine auxotrophs of *N. crassa*, *B. subtilis* and *Esch. coli* can be divided into three groups according to the compounds which they can utilize: (1) only isoleucine, (2) α,β-dihydroxy-β-ethylbutyric acid or isoleucine, (3) iso-

leucine, α,β-dihydroxy-β-ethylbutyric acid, α-aminobutyric acid, α-ketobutyric acid or threonine. Hence, the metabolism of isoleucine, the sulphur amino-acids and threonine is closely related and may proceed from a common C_4-precursor. Isoleucine may arise from the latter by the addition and reduction of an acetyl group [1*b*] (see Fig. 5.3). The keto-acids of valine and isoleucine have been identified in the culture filtrate of an isoleucinless mutant of *Esch. coli* [47].

Isotopes

Isotopes of carbon are proving useful in tracing the origin of the various carbon atoms of amino-acids, and their application to the study of syntheses in micro-organisms (*Torulopsis utilis*, *Esch. coli*, *N. crassa*) is mainly due to Ehrensvard and his colleagues [6, 12, 13]. By using acetate as a sole source of carbon and labelling the carbon in the two positions with different isotopes ($C^{13}H_3C^{14}OOH$), it is possible to determine whether a particular C atom is derived more or less directly from the CH_3 or the COOH group. With the yeast *T. utilis* adapted to growth on acetate, it was found that two acetate carboxyl groups were liberated as respiratory CO_2 for every methyl group. After hydrolysing the yeast with acid, the amino-acids were isolated by means of electro-dialysis and chromatography on ion exchange resins. The ratio of C^{14} to C^{13} in the carboxyls of most of the amino-acids was the same as that in the respiratory CO_2, thus demonstrating that CO_2 fixation had taken place. In general, all the alpha-C atoms and many of those in the side chains were derived from the methyl group of the acetate. It was concluded that glutamic acid is a precursor of arginine (cf. p. 70) and that lysine is synthesized by the head to tail condensation of acetyl radicals. When $NH_2.CH_2C^{14}OOH$ was used as a sole N source the C^{14} was finally located mainly in the glycine, serine and proline of the proteins. Thus in *Torulopsis*, as in bacteria (p. 149) and animals [cf. 14], glycine is a precursor of serine. By growing cultures of *Sac. cerevisiae*, *T. utilis* and *Ps. fluorescens* in the presence of $HC^{14}OOH$, it has been shown

that the C in position 2 of the imidazole ring of histidine is derived exclusively from formate [33, 44].

Isotopically labelled compounds such as amino-acids can be readily prepared by isolating them from organisms which have been grown in media containing substances enriched with the appropriate isotope [2]. Non-exacting organisms are especially useful since they utilize simple substances (HCO_3^-, NH_4^+, SO_4^{--}) as sources of C, N and S and such substances enriched with the appropriate isotopes are readily available.

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CHAPTER VI

ABSORPTION OF AMINO-ACIDS BY MICRO-ORGANISMS

THE amino-acids required for growth are either synthesized by the organism itself or derived from its environment. Consequently the mechanisms controlling the absorption of amino-acids are of fundamental importance, especially to exacting organisms who must perforce rely entirely on their environment for supplies of compounds which they are unable to synthesize. The factors influencing the passage of amino-acids into and out of micro-organisms, particularly those exacting towards amino-acids, have been investigated by Gale and his colleagues [6]. Whilst using the bacterial decarboxylases [4] (p. 27) for the analysis of the amino-acid composition of bacterial proteins [3], Gale found that Gram-positive bacteria contained large amounts of free amino-acids. This discovery arose from the fact that hydrolysates of whole cells, and not isolated proteins, were being analysed, and it was realized that serious errors would result if there were appreciable amounts of free amino-acids in the experimental material prior to hydrolysis. Such amino-acids might be adsorbed on to the cell surface or be present inside the cells. In order to determine the significance of these possibilities, washed cells of *Strep. faecalis* were disintegrated by shaking with glass beads, a procedure which ruptured the cells but caused no significant degradation of the proteins. Analysis of the disintegrated cells revealed the presence of relatively high concentrations of certain amino-acids (cf. columns *b* and *a*—*b* in Table 6.1). Although the latter were also found in disintegrated cells of *Staph. aureus*, there were no significant amounts in those of two Gram-negative organisms, *Esch. coli* and *Aerobacter aerogenes* [5]. Further experiments showed that the greater proportion of these amino-acids was located inside the cells.

There was therefore an indication that only Gram-

positive bacteria contained free amino-acids. Moreover, since many amino-acid exacting organisms are also Gram-positive, their presence might be the outcome of mechanisms enabling such organisms to absorb essential amino-acids

TABLE 6.1

THE AMINO-ACID COMPOSITION OF STREP. FAECALIS CELLS [5]

Results expressed in terms of amino-acid N as % total N

Amino-acid	Acid hydrolysate (*a*)	Disintegrated cells (*b*)	Combined amino-acid (*a*−*b*)
L(+)-Lysine	11·30	2·90	8·40
L(+)-Arginine	5·19	0·10	5·09
L(+)-Glutamic acid	5·60	1·74	3·86
L(−)-Histidine	2·84	0·70	2·14
L(+)-Ornithine	1·48	1·27	0·21
L(−)-Tyrosine	0·81	0·0	0·81

from their environment. In view of the potential significance of these observations, Gale decided to undertake a series of more detailed investigations. Apart from the intrinsic value of the results these experiments provide a valuable example of one type of approach to microbiological problems.

Procedure for assaying internal amino-acids

A thick suspension (about 30 mg.dry wt./ml.) of washed cells of the organism is prepared and the amount of external amino-acid is determined by adding the appropriate decarboxylase preparation to a sample of this suspension. The total amount of free amino-acid, i.e. the amount inside the cells plus that outside, is determined by adding the decarboxylase to a sample of the suspension which has been previously heated at 100° C. for 15 minutes in order to disrupt the cells. The amount of amino-acid inside the cells is then readily calculated by subtracting the former result from the latter. Concentrations are expressed in terms of either the amount of amino-acid in a specified dry weight of cells, or the amount per millilitre of cell volume or 'internal free-space'. The volume occupied by the cells was

obtained by centrifuging samples of the whole cell suspension in graduated tubes and the volume of the cellular constituents was calculated by assuming that they were mainly proteins whose specific volume is 0·70. Subtraction of the volume of the cellular constituents from the volume of an equivalent amount of whole cells yields a value for the 'internal free-space', i.e. the internal environment [5].

The amino-acid decarboxylases are enzymes of high specificity (p. 27), consequently this procedure will estimate only amino-acids which are initially free in the cells or which are liberated from compounds that are easily decomposed during the assay procedure, e.g. by heat or by other enzymes present in the decarboxylase preparations. Whilst enzymic decomposition during the assay procedure has not been ruled out, it is unlikely that they arise by the decomposition of heat-labile compounds since the same amount of amino-acid is found in cells disrupted by heat as in those made permeable by treatment with tyrocidin or phenol [13]. In the absence of evidence to the contrary the internal amino-acids are regarded as being 'free' in the sense that they are chemically uncombined.

Internal amino-acids in Gram-positive organisms

Washed suspensions of twenty-seven organisms comprising thirteen genera were prepared from cultures which had been grown on a medium rich in amino-acids. The experiments were restricted to the six amino-acids for which specific decarboxylases were then known. Free amino-acids were found only in Gram-positive organisms, yeasts as well as bacteria, and none were detected in Gram-negative bacteria. The yeasts contained high concentrations of all six of the amino-acids whereas the bacteria contained only lysine and glutamic acid in appreciable amounts [18].

Absorption of amino-acids by washed cells

The factors controlling the absorption of lysine and glutamic acid have been studied in experiments with washed cell suspensions of *Strep. faecalis* and *Staph. aureus* (*Micrococcus pyogenes* var. *aureus*), both of which are exacting

towards amino-acids. Cells which initially contain negligible amounts of amino-acids (i.e. 'amino-acid deficient cells') are especially suitable for this type of experiment and they were obtained from cultures grown on a liquid medium in which the concentration of amino-acids was just sufficient for growth. Such cultures were harvested near to the cessation of active cell division since the ability to absorb amino-acids was observed to decline appreciably in the stationary phase. The amino-acid deficient cells were suspended in an inorganic salt medium (pH 7·2) to which other substances (amino-acids, glucose, etc.) were added as required. At the end of the experimental period the cells were collected by centrifuging, washed, made into a thick suspension and the internal concentration of the amino-acid determined. Unless stated to the contrary the results given below apply to *Strep. faecalis.*

Uptake of lysine [5]

As soon as lysine was added to the experimental system it began to pass into amino-acid deficient cells of *Strep. faecalis*, and after about 15 minutes equilibrium was reached and there was no further increase in the internal concentration. This uptake of lysine occurred rapidly at 37° C. and was still appreciable at 4° C. The rate of appearance of lysine inside the cells was approximately directly proportional to its concentration in the external medium (Fig. 6.1), and the Q_{10} over the range of 20°–30° C. was 1·4. From these facts it may be deduced that lysine is entering the cells by a process of diffusion. However, this uptake did not represent simple equilibration between lysine-deficient cells and a lysine-rich environment, since at equilibrium the internal concentration of the amino-acid was from three to twenty times greater than the external concentration, the ratio of internal to external concentration being inversely related to the latter. In other words, the cells were accumulating lysine against a concentration gradient (Fig. 6.2). The rate of lysine absorption increased as the hydrogen-ion concentration of the salt medium was decreased to pH 9·5, and since the isoelectric point of lysine is 9·47, it is feasible that

lysine most readily enters the cells as the zwitterion. When the cells fermented glucose, the uptake of lysine was depressed but could be restored, although not completely, if glutamic acid was also added to the system. The factors influencing the outward migration of lysine were also of importance, if only to show that washing the cells prior to

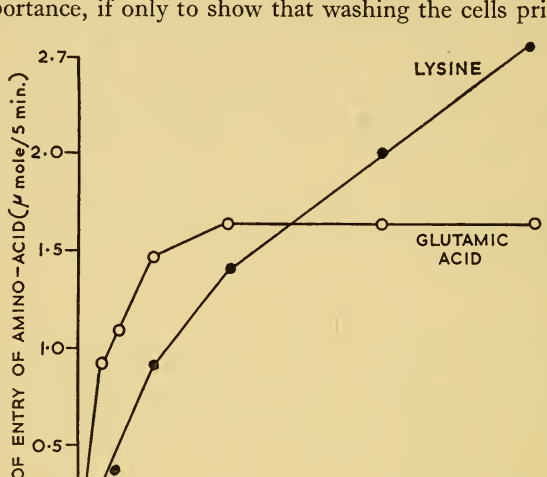

FIG. 6.1.—Effect of external concentration of L-lysine (●) and L-glutamic acid (○) on the rate of entry of the amino-acid into *Strep. faecalis*

assay did not remove any of the internal amino-acids. Cells containing large amounts of lysine, glutamic acid and probably several other amino-acids were obtained from cultures grown in the presence of a tryptic digest of casein. When these 'amino-acid rich cells' were incubated in an amino-acid free salt medium, there was no outward migration of either lysine or glutamic acid unless the cells were fermenting glucose.

More recent experiments have served to emphasize that the previous history of the cells and the presence of other amino-acids profoundly influence the inward as well as the outward migration of lysine and glutamic acid. Cells containing only lysine in large amounts were obtained by

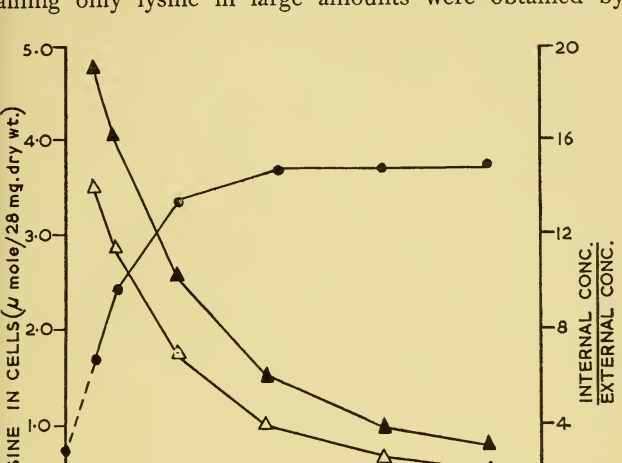

FIG. 6.2.—Effect of external concentration of lysine on (*a*) internal concentration of lysine (●) and (*b*) the ratio of internal concentration of lysine to external concentration: in ratio calculations internal concentrations expressed in terms of volume of intact cells (△) and volume of intact cells less that of solid debris (▲). *Strep. faecalis* suspended in lysine solutions for 3 hr. at 4° C. [5]

incubating a washed suspension of amino-acid deficient cells in the presence of lysine [16]. When these cells were transferred to an amino-acid free environment (0·15 M.-NaCl or Na_2HPO_4 at 37° C.) lysine migrated out into the surrounding medium and, in contrast to the previous experiments with amino-acid rich cells, the migration occurred in

the absence of glucose fermentation. It may be concluded that the presence of high concentrations of other amino-acids in the internal environment promotes the retention of lysine inside the cells and that their influence is overcome by glucose fermentation. The converse is also true, since the presence of acidic or basic amino-acids in the *external* environment retarded the uptake of lysine by amino-acid deficient cells in the absence of glucose. The effects of orthophosphate on the outward migration of lysine have been investigated [16], but the results are difficult to interpret and the reader is referred to the original paper.

From all these results, it may be deduced that the accumulation of lysine by *Strep. faecalis* involves a diffusion mechanism which is independent of metabolic energy and may be due to the establishment of a Donnan type of equilibrium. Such a hypothesis is supported by the observation that, when suspended in an amino-acid free medium, lysine migrated out of cells which contained only this amino-acid in large amounts. However, it must not be assumed that the mechanism of lysine absorption is the same in all Gram-positive organisms. Although similar results were obtained with *Staph. aureus*, the uptake of lysine by *Sac. cerevisiae* was dependent on metabolic energy [6] and is therefore comparable to the uptake of glutamic acid by *Strep. faecalis* and *Staph. aureus*.

Uptake of glutamic acid [5]

Irrespective of the pH of the inorganic salt medium there was no absorption of glutamic acid by the amino-acid deficient cells unless glucose or a tryptic digest of casein was also added. Hence, unlike lysine, the uptake of glutamic acid appeared to be an endergonic process utilizing metabolic energy made available by the fermentation of glucose or by the metabolism of amino-acids. Arginine promoted the absorption of glutamic acid, although not as efficiently as glucose, and the fact that the organisms possess arginine dihydrolase (p. 27) may be of some significance. In the presence of glucose, glutamic acid was not only absorbed but also concentrated in the cells, and the relationships

between the internal and external concentrations at equilibrium were similar to those found for lysine (cf. Fig. 6.2). But, in contrast to lysine, the rate of uptake was not directly proportional to the external concentration (Fig. 6.1) and the relationship was reminiscent of that between the rate of an enzyme reaction and the concentration of the substrate. The Q_{10} over the range 20°–30° C. was 1·94, which is close to 2·0 and therefore indicative of a chemical and presumably of an enzymic reaction.

Various inhibitors of intermediary metabolism, such as cyanide and iodoacetate, have no effect on the uptake of lysine, but any substance inhibiting fermentation also inhibits the uptake of glutamic acid. However, it is possible to separate the processes of energy production from those of energy utilization by preferential inhibition of the latter. This may be accomplished by using a substance such as 8-hydroxyquinoline which in low concentrations inhibits the uptake of glutamic acid without affecting fermentation, although higher concentrations inhibit the latter as well [7]. Since 8-hydroxyquinoline is a chelating agent, the conclusion was drawn that cations played an important role in glutamic acid absorption. *Staphylococcus aureus* was used in these experiments and the problem was further investigated by growing the organism in the amino-acid poor medium from which one or more cations had been removed. The ability to absorb and concentrate glutamic acid was seriously impaired only in cells harvested from media deficient in Mg^{++} or Mn^{++}. Whilst there is insufficient data for deciding which of these ions is the natural activator of the glutamic acid absorption mechanism, it is pertinent to note that both of these cations are frequently found as co-factors of enzymes associated with phosphorylation.

In view of the dependence of the process on metabolic energy it is conceivable that glutamic acid passes through the cell wall in the form of a compound whose synthesis is endergonic (e.g. as glutamine, glutathione, or a phosphorylated derivative), and having passed through the cell wall, this compound is reconverted to the free acid. Glutamine, glutathione and glutamylglutamic acid all failed to enter

the cells unless glucose was also added to the experimental system. On the other hand, α,γ-diethylglutamate and N-phosphorylglutamic acid were absorbed in the absence of glucose and gave rise to internal glutamic acid [8]. Hence it is possible that glutamic acid passes through the cell wall in a phosphorylated form, and irrespective of whether the phosphate group is on the amino group or on one or both of the carboxyl groups, the synthesis of such a compound would be endergonic. But, the evidence in favour of this hypothesis is far from conclusive. Many cells are known to be impermeable to phosphorylated compounds and the Q_{10} for the uptake of N-phosphorylglutamic acid was intermediate between that for free diffusion and for an enzymic process.

When amino-acid deficient cells of *Staph. aureus* were incubated with glutamic acid and glucose together with mixtures of other amino-acids, the accumulation of free glutamic acid was reduced and sometimes ceased. This was the outcome of a marked reduction in the amount absorbed and also an increase in the cellular combined glutamic acid. If the concentration of glutamic acid in the external medium was very much greater than that of the other amino-acids, assimilation into cell substance was suppressed and some accumulation of the free acid did take place. Evidently whether glutamic acid entered the cells and accumulated as the free acid or whether it was converted into a combined form, depended on the ratio of glutamic acid to other amino-acids in the external medium. The presence of single acids such as aspartic, cysteine, glycine, serine and alanine produced a marked decrease in the rate of glutamic acid accumulation. Valine, leucine and isoleucine caused a small increase in the latter, whilst all the other amino-acids examined had no effect. Aspartic acid acted as a competitive inhibitor, but this explanation did not apply to cysteine and alanine, the addition of which led to the synthesis of extracellular peptides containing glutamic acid [10, 11].

There was a small leakage of glutamic acid when cells of *Staph. aureus* containing large amounts of several amino-acids were incubated in an amino-acid free medium: the

addition of glucose completely suppressed this outward migration. On the other hand, there was no outward migration of glutamic acid from *Strep. faecalis* unless glucose was present [5].

The uptake of histidine and aspartic acid by *Strep. faecalis* is also dependent on metabolic energy [5] and in *Sac. cerevisiae* this applied to all of the amino-acids investigated [19, 6].

Mechanism of amino-acid absorption

Whilst there are unaccountable differences even between somewhat similar organisms such as *Strep. faecalis* and *Staph. aureus* (cf. the action of inhibitors in 6, 9), there is sufficient data to warrant a discussion of the mechanisms which may be operative in the absorption of amino-acids. This topic is of particular interest since the physical and chemical structure of the barrier separating the interior of the bacterial cell from the external environment is now being actively studied by several workers [15, 17, 21]. Moreover, amino-acids exist in solution as ions, consequently their mode of absorption may be only one aspect of the general problem of ion transport across cellular membranes [cf. 20].

The normal electrochemical properties of a cell will be markedly altered by the absorption of ions of one species unless there is simultaneously an equivalent migration of ions either of opposite charge into the cell or of like charge out of the cell. Many experiments with plant and animal cells show that the uptake of one type of ion usually involves the concurrent transport of other ions, and it is therefore legitimate to expect that the uptake of amino-acids is likewise associated with the movement of other ions. Whether this does in fact happen has not yet been investigated, and indeed little is known about the migration of ions into and out of micro-organisms. Several plant and animal cells contain higher concentrations of particular inorganic ions than the extracellular fluids, e.g. the K^+ content of mammalian cells is greater than that of the plasma. One explanation of the unequal distribution of diffusible ions between cells and their environment has been advanced by Donnan, who

proposed that such a distribution in a system in equilibrium is the natural outcome of the presence of intracellular non-diffusible ionic substances such as proteins [2].

The experiments with *Strep. faecalis*, *Staph. aureus* and *Sac. cerevisiae* have shown that certain amino-acids can pass into the organisms against the concentration gradient and at equilibrium their internal concentration may be, and often is, greater than their concentration in the external environment. Furthermore, two distinct mechanisms appear to be operating in the absorption of amino-acids, one involving simple diffusion, e.g. the uptake of lysine by *Strep. faecalis* and *Staph. aureus*, whilst the other involves the utilization of metabolic energy, e.g. the uptake of glutamic acid. These facts may be accounted for by one of four explanations:

(i) That as a result of electrostatic attraction, the amino-acid becomes associated inside the cell with a non-diffusible ion of opposite charge, thus establishing a type of Donnan equilibrium [2].

(ii) That once inside the cell, the amino-acid is converted into a compound which cannot itself pass through the cell wall.

(iii) That the cell is permeable, not to the amino-acid itself, but to a derivative whose synthesis is endergonic.

(iv) That the migration of the amino-acid results from the movement of another ion whose formation or transport is endergonic.

Mechanism of lysine absorption

Najjar and Gale suggested that the absorption and accumulation of lysine in *Strep. faecalis* is due to the establishment of a type of Donnan equilibrium [16]. In physiological conditions of pH lysine carries an overall positive charge, consequently if only lysine is absorbed it is reasonable to suggest that this is accompanied either by the excretion of an equivalent amount of another cation such as H^+ or K^+, or by the absorption of an equivalent amount of

anion. Even though the latter takes place, there may be another mechanism which excretes an equivalent amount of either the same or a different anion together with cations to replace the lysine absorbed. It may be recalled that fermenting yeast absorbs K^+ from a medium containing KCl and replaces it by H^+ [see 20]; there is apparently no uptake of Cl^-. A possible explanation here is that metabolic energy is used to form H^+, which is then secreted into the medium in exchange for K^+, thus making it appear that the uptake of K^+ is an active process, i.e. dependent on metabolic energy. It may therefore be suggested that lysine is absorbed by *Strep. faecalis* in exchange for cellular K^+ or another cation which does not have to be formed at the expense of metabolic energy. However, recent work dealing with the accumulation of lysine showed that in *Strep. faecalis* it was accompanied by a gain in cellular K^+ (with no significant change in cellular Na^+) whereas with *Staph. aureus* it had no apparent effect on either the K^+ or Na^+ content of the cells. In *Saccharomyces fragilis*, the uptake of lysine was dependent on glucose fermentation and was accompanied by the loss of Na^+ and K^+ from the cells [1*b*] (in none of these experiments was the migration of OH^- and H^+ studied).

Mechanism of glutamic acid absorption

Having regard to the second explanation (ii) advanced above, it might be suggested that the 'free glutamic acid' of the cell is in the form of glutamine, a substance whose synthesis is endergonic and which is assayed by the decarboxylase preparation as though it is the free acid: moreover, as glutamine cannot freely diffuse into cells, it is possible that it cannot itself pass through the cell wall [5]. But only a small part of the glutamic acid of the internal environment of streptococci is in fact in this form [14]. Hence the energy associated with the uptake of glutamic acid may be used in the manner outlined in the last two explanations, iii and iv. The former of these proposes that active transport, i.e. transport dependent on metabolic energy, involves chemical

reactions between cellular constituents and the substance being transported across the cell membrane [20]. This implies that the latter contains a substance or carrier which reacts with the transported substance to form a relatively stable product which traverses the cell membrane and then either undergoes chemical decomposition or takes part in an exchange reaction, thus liberating the transported substance into the internal environment. Energy may be required for the formation of either the compound traversing the membrane or the substance which takes part in the final exchange reaction. Davies and Krebs [1*a*] have shown theoretically how metabolic energy may be utilized for the production of H^+ or OH^- from water. By analogy with their hypotheses concerning ion transfer in brain cells it may be proposed that metabolic energy is used to form an excess of OH^-, which may perhaps combine with CO_2 to form HCO_3^-, and that the bacterial cell wall contains a basic ion exchange complex (X). If the cell contains a high concentration of an anion such as OH^- or HCO_3^- and the external environment contains glutamate ions, and if it is assumed that the ion exchange complex X can move in the cell wall and thus come into contact with the external and internal environments, the following reactions can be expected to take place, the equilibrium being towards the right:

$$\text{internally: } X^+ + OH^- \rightleftharpoons X\text{-}OH$$
$$\text{externally: } X\text{-}OH + \text{glutamate} \rightleftharpoons X\text{-glutamate} + OH^-$$
$$\text{internally: } X\text{-glutamate} + OH^- \rightleftharpoons X\text{–}OH + \text{glutamate}$$

Glutamate ions will thus be transported into the cell and will be replaced in the external environment by hydroxyl ions. Eventually a steady state will be established in which exchange is still taking place, but there is no further overall increase in the internal concentration of glutamic acid. Specificity in ion transport may be due to different ions being transported by different ion-exchange complexes whose specificity properties are comparable with enzymes. The observation by Britten that intracellular glutamic

acid will exchange with extracellular isotopically labelled glutamic acid in the absence of metabolic energy can be explained if there is a carrier mechanism of this type in the cell membrane. All these theories are purely speculative and their acceptance or rejection awaits the results of further experiments. One fact of which account must now be taken is that the accumulation of glutamic acid within fermenting cells of *Staph. aureus*, *Strep. faecalis* and *Sac. fragilis* is accompanied by an increase in cellular K^+, the increase appearing to be of the order 1 gram atom K^+/mole glutamic acid [1*b*].

The full significance of the ability to absorb and accumulate amino-acids still awaits complete evaluation since only three organisms and a restricted number of amino-acids have so far been investigated. It may be remarked that a decrease in the nutritional requirements of *Staph. aureus* is accompanied by a decreased ability to accumulate amino-acids [12], but it must also be noted that *Sac. cerevisiae* is not exacting towards amino-acids and yet accumulates many of these compounds [19]. Moreover, washed suspensions of a large variety of organisms, Gram-positive as well as Gram-negative, decompose several amino-acids, the implication being that unless the appropriate enzyme is in the cell surface the amino-acid enters the cell by free diffusion.

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CHAPTER VII

PEPTIDES AND PROTEINS

MODERN concepts of protein structure are founded mainly on the results obtained by subjecting proteins of animal origin to procedures involving partial and complete hydrolysis, amino-acid analysis, ultracentrifugation, electrophoresis and X-ray diffraction analysis. From the limited data available there is, however, no reason to believe that the structure and general physical properties of the proteins of micro-organisms are in any way different from those of animals and plants. A few representative microbial proteins have been purified and in some cases crystallized, e.g. the enzymes alcohol dehydrogenase, catalase, amylase and an extracellular proteinase from *Sac. cerevisiae*, *M. lysodeikticus*, *B. subtilis* and *Strep. haemolyticus* respectively and the toxins of *Cl. botulinum* and *Cl. tetani*. Analyses of acid hydrolysates of such proteins and of whole cells by classical precipitation procedures, microbiological assay [41], the bacterial amino-acid decarboxylases (p. 27), and chromatography on ion exchange resins or paper have provided adequate evidence that the proteins of micro-organisms are composed of the L-stereoisomers of the same α-amino-acids as are those of more complex multicellular organisms.

Whilst there is no conclusive proof that D-amino-acids are constituents of proteins and it is probable that any found in acid or alkaline hydrolysates have arisen by racemization, they are not metabolically inert, and indeed several of them can be utilized by many micro-organisms [36]. The first step in their metabolism may involve deamination by a D-amino-acid oxidase (p. 11): alternatively, direct conversion to the corresponding L-isomer may be accomplished by a racemase, and at the present time two are known, specific for alanine [46] and glutamic acid [31] respectively. Both possess a prosthetic group of pyridoxal phosphate and the alanine racemase

is widely distributed [46]. Several bacterial peptides, especially those secreted into the environment, contain D-amino-acids and, in addition, amino-acids not yet found in peptide combination in animals and plants (Table 7.1). Whether these unusual amino-acids are also present in bacterial proteins remains to be proved. On the basis of their biological activity, the peptides associated with the metabolism of micro-organisms can be arranged into three groups:

(i) Peptides which are or may be co-factors in intermediary metabolism, e.g. glutathione, the folic acid factors, biocytin (p. 24), glutamine and asparagine. Though the latter three compounds are not true peptides, they can be regarded as containing the 'peptidic bond', —CO—NH— [25].

(ii) Peptides which serve as a source of amino-acids essential for growth. In natural environments, these peptides arise as the result of autolysis and the action of extracellular proteases (pp. 115–16).

(iii) Extracellular peptides. Among the bacteria, members of the Bacillaceae are especially active in the formation of this type of peptide, the majority of which possess antibiotic activity, an exception being the polypeptides of D-glutamic acid which may be attached to the organism in the form of a capsule (*B. anthracis* and *B. mesentericus*) or free in the medium (*B. subtilis* and *B. mesentericus*) [2]. The capsular material of *B. anthracis* consists of chains of glutamic acid residues linked by gamma peptide bonds together with chains linked by alpha peptide bonds. Such a capsule may confer immunity from attack by proteases of the infected host [15].

Co-factors containing peptide bonds

The available evidence indicates that glutamine and asparagine play important roles in intermediary metabolism and it is to be noted that several co-factors are known which, like glutamine, contain the γ-glutamyl radical. Glutamine may be essential for initiating the growth of certain organisms, but in many instances it is replaceable by glutamic

TABLE 7.1

ANTIBIOTIC SUBSTANCES CONTAINING AMINO-ACIDS

G+, G−, AF, =antibiotic active against Gram-positive, Gram-negative, and acid-fast bacteria respectively: ( )=only active against a few species or in high concentrations

Antibiotic substance	Source	Antibiotic activity	Notes
gramicidin	*Bacillus brevis*	G+, (G−)	cyclic peptide, components include D-valine, D-leucine and ethanolamine: neutral surface-active agent
gramicidin S	*Bacillus sp.*	G+, G−	cyclic peptide, constituents include D-phenylalanine and L-ornithine
tyrocidin	*Bacillus brevis*	G+, G−	cyclic peptide, components include D-phenylalanine: cationic surface-active agent
licheniformin	*Bacillus licheniformis*	G+, (G−), AF	polypeptide, cyclic?, 3 types, A, B and C
polymyxin	*Bacillus polymyxa*	G−	peptide, 5 types, all contain D-6-methyloctan-1-oic acid and L-α, γ-diaminobutyric acid: Polymyxin A contains D-phenylalanine and D contains D-serine
bacitracin	*Bacillus subtilis*	G+	polypeptide, cyclic?, several types: components include D- and L-aspartic acid, D-isoleucine, and D- and L-phenylalanine
subtilin	*Bacillus subtilis*	G+, G−, AF	polypeptide, components include lanthionine, and unidentified diamino acid, $C_7H_{14}N_2O_4S$
nisin	*Streptococcus lactis* gp. N	G+	polypeptide, component amino-acids include methionine, lanthionine and cystathionine
actinomycin	*Streptomyces antibioticus*	G+, G−	quinone-peptide conjugate; actinomycin A contains sarcosine, L-*N*-methylvaline and D-valine, C also contains D-alloisoleucine
vinactin	*Actinomyces vinaceus*	G+, G−, AF	peptide, several types, vinactin B contains sarcosine
viomycin	*Streptomyces floridae*	G+, G−, AF	conjugated peptide, contains α,β-diaminopropionic acid
penicillin	*Penicillium notatum*	G+, (G−)	hydrolysis yields D-β-thiovaline

acid, though usually only if much larger amounts are supplied. When small inocula are used, the glutamine requirements of *Strep. haemolyticus* (groups A and C) are absolute and cannot be replaced in this manner [10]. Streptococci decompose glutamine to glutamic acid and ammonia only when they are fermenting glucose, and the presence of small amounts of glutamine was observed to stimulate the fermentation of glucose by washed cell suspensions of all the streptococci examined, irrespective of their glutamine requirements during growth [27]. This stimulation was far greater than that produced by an equivalent amount of ammonium glutamate, and the more dilute the suspension, the greater the stimulation. Glutamine here appears to function by restoring the intracellular concentration of a diffusible co-factor to an optimal value. Similar results were later obtained with *Ln. mesenteroides*, *Lb. arabinosus* [45] and *Cl. tetani* [24]. Unlike the streptococci, *Pr. morganii* [26], *Esch. coli*, and *Cl. welchii* [18] are able to hydrolyse the amide group of glutamine in the absence of glucose fermentation. Asparaginase—the enzyme system catalysing the hydrolysis of the amide group of asparagine—is widely distributed in fungi, yeasts and bacteria [cf. 50], and for this reason asparagine is frequently incorporated in media as a convenient source of readily available carbon and nitrogen. Asparaginase has been found, for example, in autolysates of *Ps. pyocyanea*, *Esch. coli*, *B. subtilis* and *Pr. vulgaris*. Asparagine is an essential nutrilite for some strains of *Ln. mesenteroides* and *Strep. lactis*.

Glutamic acid is also a constituent of the folic acid factors, substances essential for the growth of *Strep. faecalis R* and *Lb. casei* and of key importance in the metabolism of all organisms. These factors contain a pterin linked to *p*-aminobenzoic acid (PAB) which is in turn coupled through the amino group to one or more residues of glutamic acid (one in synthetic folic acid, three in the fermentation *Lb. casei* factor and seven in vitamin B_c conjugate). The linkages between PAB and glutamic acid and between the various glutamic acid molecules probably involve the γ-carboxyl groups of the amino-acid. Whilst the enzyme systems in which folic

acid participates have yet to be isolated, there are good reasons for believing that it is implicated in the synthesis of certain amino-acids, purines and pyrimidines (pp. 146–51).

Glutathione (GSH), the first peptide to be assigned the function of a co-factor in intermediary metabolism, was discovered and isolated by Hopkins from yeast and various animal tissues. Little is known about the distribution of this peptide in bacteria [4] and its isolation from these organisms has not yet been reported. The chemical synthesis of GSH by Harington and Mead provided conclusive proof that it was γ-glutamylcysteinylglycine. Ever since GSH was known to contain a thiol group, it has been postulated that GSH entered into cellular oxido-reduction reactions:

$$2\text{GSH} \longrightarrow \text{GS—SG} + 2\text{H}$$

In the presence of glutathione reductase, an enzyme found in yeast, plants and animals, oxidized glutathione will accept hydrogen from reduced TPN but not DPN: the reverse reaction has not yet been demonstrated [6]. The activity of many enzymes is inhibited by substances which react with or oxidize thiol groups, and it is therefore possible that GSH is part of the mechanism whereby these enzymes are maintained in or brought into an active state *in vivo*. Glutathione takes an active part [34] in the conversion of methylglyoxal to lactic acid, a reaction catalysed by the enzyme system glyoxalase, found for example in *Esch. coli* and *Sac. cerevisiae*. Racker and Krimsky [35] have shown that GSH is tightly bound to the enzyme triosephosphate dehydrogenase, and they suggest that a thiol ester of 3-phosphoglyceric acid is an essential intermediate stage in the formation of 1:3-diphosphoglyceric acid (cf. the role of Co.A in the pyruvic oxidase system). Certain reactions in which GSH participates as a substrate rather than as a co-factor have recently aroused great interest because of their potential significance in the synthesis of peptides and proteins *in vivo*. Working with cell-free preparations of sheep kidney, Hanes, Hird and Isherwood [16] have demonstrated that the γ-glutamyl group of GSH and other γ-glutamyl peptides (but not glutamine) can be transferred to peptides or to amino-acids,

i.e. the carboxyl moiety of a peptide bond can be transferred to a suitable amino acceptor. Thus incubation of GSH with phenylalanine or tyrosine resulted in the formation of γ-glutamylphenylalanine and γ-glutamyltyrosine respectively. Evidence that new peptides had been synthesized was first obtained by paper chromatography, and some of these compounds have now been isolated and characterized. The term transpeptidation has been applied to such transfer reactions and similar results were later obtained with *Pr. vulgaris* [37]. Hanes *et al.* noted that prolonged incubation tended to produce complete hydrolysis of all the peptides in the experimental system: it is therefore possible that these transpeptidation reactions are catalysed by the intracellular proteases and indeed various proteases, like several other hydrolytic enzymes, are known to be capable of performing transfer reactions (pp. 104–5).

Utilization of peptides by micro-organisms

Information concerning the utilization of peptides comes mainly from the response of nutritionally exacting organisms to peptides containing an amino-acid essential for growth. The majority of these studies have been performed with non-proteolytic species and provide evidence that hydrolysis by extracellular proteases is not an obligatory step in the utilization of simple peptides. Using four mutants of *Esch. coli*, exacting towards phenylalanine, tyrosine, proline and leucine respectively, Fruton and Simmonds compared growth in the presence of simple dipeptides containing the required amino-acid with that in the presence of the free acid [12]. Peptides of phenylalanine or tyrosine were as effective as equimolecular amounts of the uncombined acids and there was little or no difference in the growth curves. It was concluded that prior to utilization, these peptides were hydrolysed by intracellular peptidases at a rate which did not limit growth. *Lactobacillus arabinosus* and *Ln. mesenteroides* likewise utilize dipeptides of glutamine as readily as free glutamine or glutamic acid [45]. The leucineless *Esch. coli* mutant grew at the expense of peptides containing leucine, but although the rate of growth in the log phase and

the total amount of growth were equivalent to those on uncombined leucine, the length of the lag period was increased in proportion to the concentration of the peptide in the medium. This effect was not due to time being required for the formation of an adaptive enzyme. In experiments with *Lactobacillus delbruckii*, *Lb. casei* and *Strep. faecalis*, other workers have observed that the utilization of di- and tripeptides serving as sources of valine or leucine was affected by the position of the amino-acid in the peptide, the nature of adjacent amino-acids, and in some instances the composition of the medium [1, 23]. Unlike the examples described so far, the total amount of growth of the proline requiring *Esch. coli* mutant was greater when the amino-acid was supplied in the form of a dipeptide, yet the rate of growth was not affected. A possible explanation is that enzymes which decompose amino-acids are unable to attack those bound in peptides, consequently if as a result of peptidase activity an amino-acid gradually becomes available over a period of time, a greater proportion will be used in anabolic systems than if it is all initially present in the free state. Two other observations support such a conclusion. With *Strep. faecalis*, an organism with an active arginine dihydrolase system (p. 26), the same amount of growth was produced by appreciably less arginine when it was supplied in the form of small peptides. Similarly if the organism developed an active tyrosine decarboxylase, dipeptides of tyrosine evoked greater growth than an equivalent amount of free tyrosine [20]. In a medium containing D-alanine in place of pyridoxin, *Lb. casei* becomes exacting towards dipeptides containing L-alanine, because the D-isomer inhibits the normal utilization of uncombined L-alanine, another essential nutrilite. High concentrations of glycine prevent the utilization of D-alanine, but in the presence of pyridoxin, neither glycine nor D-alanine is inhibitory and there is no requirement for L-alanyl peptides [21].

Unidentified growth factors believed to be peptides

The growth of several nutritionally exacting bacteria appears to be dependent on, or is stimulated by, unidentified

substances which are believed to be peptides. Before attempting to decide whether such requirements are absolute it should be noted that in many instances the nature of the response is determined by the composition of the medium and the period for which the cultures are incubated [22]. Strepogenin is the name given to acid-labile material present in enzymic digests of proteins and required for the growth of certain streptococci and *Lb. casei* in synthetic media. The strepogenin activity of protein digests cannot be explained solely in terms of their content of glutamine or asparagine since neither of these substances replaced the digest factor for a strain of *Strep. faecalis* [48] and the response of *Lb. casei* to glutamine was different from that to strepogenin [47]. Moreover, the activity of both glutamine and GSH, unlike strepogenin, was destroyed by autoclaving. From their experiments with digests of crystalline insulin, Sprince and Woolley concluded that glutamic acid and glycine are two components of strepogenin, and of a large number of synthetic peptides, only tripeptides exhibited any activity, serylglycylglutamic acid being the most effective, though none was as active as the protein digest factor(s). Strepogenin was antagonized by peptides containing aspartic acid and also by lycomarasmin, a peptide secreted by *Fusarium lycopersici* and responsible for the wilting of tomato plants. Lycomarasmin is composed of asparagine, glycine and α-hydroxyalanine, with the two latter sharing a common nitrogen atom, but the detailed structure is not known. From these various experiments, it seems likely that strepogenin contains a γ-glutamyl residue and perhaps functions as a stable source of such groups for the synthesis of various co-factors. It is worthy of note that organisms responding to strepogenin also readily decompose glutamine, and this may be another example of an essential nutrient being utilized more efficiently when it is supplied in the form of a peptide. The connection between strepogenin and the dicarboxylic amino-acids and their amides is, however, far from clear [cf. 42], and it is not yet possible to account for all the experimental results. *Gravis* and *intermedius* strains of *Corynebacterium diphtheriae* also require peptide

growth factors of unknown structure, and some similarities with strepogenin are indicated [5].

Synthesis of peptides and proteins

Owing to the paucity of available information the synthesis of peptides and proteins can only be discussed in general terms. Several suggestions have been made as to the mode of formation of peptide bonds and the three most likely mechanisms are those concerned with (i) the direct utilization of metabolic energy, (ii) transfer reactions and (iii) the reversal of proteolysis.

Calculations based on the synthesis of dipeptides in water and on the hydrolysis of dipeptides to ionic products have shown that the free energy associated with the peptide bond is in the range 420 to 3,000 calories [8, 25]. Though the precise amount may be the subject of dispute, it is clear that the *de novo* synthesis of a peptide bond is endergonic, and it is reasonable to suggest that in biological systems ATP functions as a source of energy for the synthesis of peptide bonds. Glutathione, glutamine, acetylsulphanilamide and hippuric acid are all simple compounds containing peptide or peptidic bonds and the synthesis of each of these substances was first observed in actively respiring preparations of animal tissues. Any condition inhibiting respiration and in consequence the production of energy, also inhibited synthesis. The recognition of ATP as a biological carrier of energy enabled the experimental systems to be greatly simplified and studies with non-respiring cell-free preparations revealed that synthesis was dependent on the presence of ATP. It was observed that a new co-factor, termed Coenzyme A, played an important role in the synthesis of acetylsulphanilamide. Coenzyme A is now known to contain β-mercaptoethylamine and to function *in vivo* as a carrier of acetyl and other acyl groups by virtue of its ability to form thiol esters. Studies with cell-free systems have shown that in addition to being synthesized from acetate in the presence of ATP, acetyl-Co.A can also be formed directly from an acetyl donor of suitable potential without the intervention of ATP, e.g. from pyruvate by the pyruvic oxidase system

[see 32]. Dried cell preparations of *Clostridium kluyveri* catalyse the acetylation of amino-acids by acetylphosphate, a reaction which, although analogous to the synthesis of acetylsulphanilamide, only occurs in the presence of 0·1 M. cyanide. Acetylated amino-acids contain a peptidic bond, and it is feasible that peptides can be formed by transfer reactions in which the acetyl group is replaced by an amino-acid [40]. Cell-free extracts of *Staph. aureus* [9] and a number of other bacteria [13] and *Sac. cerevisiae* catalyse the synthesis of glutamine from glutamic acid and ammonia in the presence of ATP and Mg^{++} or Mn^{++}. For each mole of amide synthesized, one mole of inorganic orthophosphate is liberated, and if ammonia is replaced by NH_2OH the product is γ-glutamylhydroxamic acid. By analogy with the role of Co.A as a carrier of acetyl groups in the synthesis of acetylsulphanilamide, it is tempting to suggest that glutamine synthesis involves the formation of the corresponding γ-glutamyl compound, but all attempts to obtain supporting evidence have failed. Though both glutamine and glutathione contain the γ-glutamyl radical, there is no proof that their synthesis involves a common enzyme system or that the former participates in the synthesis of the latter. When incubated with ATP, K^+, Mg^{++}, phosphate and hexose diphosphate, cell-free extracts of *Esch. coli* synthesized GSH from glutamic acid, glycine and cysteine [37]. Whilst earlier and similar experiments with preparations of rat liver showed that the enzymes which synthesized GSH are distinct from those catalysing hydrolysis, the individual steps in the biosynthesis are not yet known; the first one may be the formation of γ-glutamylcysteine [38].

In addition to synthesis at the direct expense of metabolic energy, new peptide bonds may be formed by transfer (transpeptidation) reactions of the type:

$$XCO.NH.R+NH_2R^1 \rightleftharpoons X.CO.NH.R^1+NH_2R$$

where XCOOH, $R.NH_2$ and $R^1.NH_2$ represent amino-acids or peptides. Such reactions do not result in an overall increase in the number of peptide bonds, and since the type

and number of bonds in the products is the same as in the reactants, they proceed with little overall change in free energy and are therefore independent of the availability of metabolic energy. Several typical proteolytic enzymes are known to catalyse transpeptidation reactions, and furthermore, to lengthen a peptide chain by the direct coupling of peptides. Thus, with chymotrypsin [11]:

benzoyl-L-tyrosinamide+glycinamide ⇌
benzoyl-L-tyrosylglycinamide+NH_3
benzoyl-L-tyrosine+glycinamide ⇌
benzoyl-L-tyrosylglycinamide+H_2O

It is therefore possible, as suggested many years ago, that the action of the proteases is reversible and that in the appropriate conditions they catalyse the synthesis and not the hydrolysis of peptides and proteins. Transpeptidation reactions involving GSH have already been described (p. 99). Because cysteinylglycine is readily hydrolysed by cellular enzymes and yet is relatively stable when combined, as in glutathione, Hanes and his colleagues proposed that the attachment of a γ-glutamyl radical to a peptide confers resistance to hydrolysis by intracellular proteases, and in consequence, synthesis is favoured and the peptide chain can be gradually lengthened by successive transfer reactions. Several bacteria catalyse exchange reactions between the amide group of asparagine or glutamine and hydroxylamine or isotopically labelled NH_4^+: with NH_2OH, such reactions lead to the formation of aspartyl- and glutamyl-hydroxamic acid respectively [45]. Whether the amide group can likewise be replaced by an amino-acid or peptide is not known. Protein synthesis may therefore be visualized as a stepwise process beginning with glutathione, and possibly glutamine, as a source of peptide bonds synthesized at the expense of energy derived from ATP, and by means of transpeptidation reactions the amino-acid components of such bonds are subsequently altered so as to form peptides from which specific proteins are synthesized by further transfer and coupling reactions [11].

The mechanisms operative in the formation of peptide bonds are only one aspect of protein synthesis; it is also necessary to consider (i) the means whereby the correct sequence of amino-acids is attained in a peptide chain, (ii) if the protein molecule comprises more than one peptide chain, how such chains are linked together and (iii) the spatial arrangement of the amino-acids and the peptide chains. Major advances in solving these problems await the determination of the structure of specific proteins (cf. Sanger's recent elucidation of the amino-acid sequence in the peptide chains of insulin). Investigations of protein synthesis in animal tissues have been mostly confined to an examination of the conditions in which isotopically labelled amino-acids are incorporated into material precipitated by trichloracetic acid, i.e. presumably bound in proteins or polypeptides. Such experiments have proved little except that incorporation is associated with the utilization of metabolic energy, and great care is required in making deductions from the observed results [49]. A more direct approach is provided by studies of the synthesis of a specific protein, e.g. an enzyme whose activity can be estimated and used as an index of concentration. Evidence has gradually accumulated to the effect that at least in certain cases the adaptive formation of enzymes is the outcome of *de novo* protein synthesis rather than the mere subtle modification of existing proteins, i.e. enzymes or 'enzyme precursors' [30]. If this is true, then the formation of adaptive enzymes would appear to offer a most promising field for studying protein synthesis in micro-organisms. Concentrations of 2:4-dinitrophenol and azide which, although not affecting respiration and the fermentation of carbohydrates, inhibit the uptake of inorganic phosphate and in consequence the synthesis of energy-rich phosphate bonds, also inhibit adaptive enzyme formation and the incorporation of isotopically labelled amino-acids into peptides and proteins.

Certain observations indicate that the assembling of the constituent amino-acids is a preliminary step in the synthesis of a protein. For example, the synthesis of the adaptive enzyme nitratase in washed cells of *Esch. coli* [33] and of

amylase by pigeon pancrease is enhanced by the addition of amino-acids, and the greater the *number* of amino-acids, the greater their effect. Gale has recently studied the effect of other amino-acids, and of purines and pyrimidines on the absorption, accumulation and further metabolism of glutamic acid by *Staph. aureus*, and obtained evidence that an increase in cellular combined glutamic acid is indicative of the synthesis of new protein [14]. Protein synthesis only occurred when the cells were suspended in a medium which contained, in addition to glutamic acid and glucose, all the amino-acids to which *Staph. aureus* is exacting.

Studies of actively dividing embryonic cells and cells engaged in rapid protein synthesis led Caspersson and independently Brachet to propose that protein synthesis is preceded by the synthesis of pentose nucleic acids and that these substances then participate in and control the synthesis of proteins. By using the ultraviolet light microscope technique (p. 130), Malmgren and Hedén measured the nucleic acid content of cells at various stages during the growth of cultures of *Esch. coli* and *Bacillus cereus*. Their results indicated that the lag phase was a period of intense nucleic acid synthesis and in consequence the cellular concentration of nucleic acid reached a maximum during the early part of the lag phase: thereafter it gradually declined and became minimal during the stationary phase. Malmgren and Hedén concluded that Caspersson's and Brachet's hypothesis also applied to bacteria and that a culture only passed out of the lag phase when a critical intracellular concentration of nucleic acid has been attained [28]. Other workers using *Staph. aureus* have provided further evidence in support of this conclusion [14, 29]. The rate of protein synthesis by washed cells of *Staph. aureus* can be directly correlated with the nucleic acid content of the cells at the time of harvesting [14]. During the growth of bacterial cultures it is the pentose nucleic acid content of the cell which alters: the desoxypentose nucleic acid content remains approximately constant [29]. It is interesting to note that after irradiation with ultraviolet light, bacteria are unable to develop the usual adaptive increase in activity when they are incubated in the

presence of the specific substrate [44], and that the action spectrum for light of different wavelengths resembles the absorption spectra of the nucleic acids [43].

Such experiments have naturally focused attention on the possible role of the nucleic acids in protein synthesis and so far two theories have been proposed: one suggests that the bond energy of the nucleic acid phosphate groups is used for the synthesis of peptide bonds, whilst the other regards the nucleic acids as being the fundamental components of the organized systems controlling the sequence in which amino-acids are joined together. Proteases appear to possess well-defined specificity with regard to the peptide bonds they attack (p. 113), and if they can in fact function synthetically the same specificity is to be expected in the reverse reactions. If such enzymes can be organized so that they act in a predetermined sequence, a mechanism can be envisaged which possesses the ability to synthesize a peptide chain with the required serial arrangement of amino-acid residues. Since nucleic acids differ in their composition and structure and readily form complexes with proteins, it is possible that different nucleic acids combine specifically with different proteins. Hence an organized system of nucleic acids may provide a framework on to which enzymes are adsorbed in a particular order, with the result that they then direct the synthesis of a specific substance [17]. It is also feasible that the nucleic acids are structures on to which amino-acids rather than enzymes are adsorbed, and that differences in nucleic acid structure give rise to different sequences of amino-acids [3]. If there is any truth in such speculations, it is to be expected that disruption of nucleic acid metabolism will immediately result in the cessation of protein synthesis. It could therefore be argued that the effects of ultraviolet light described above are due to disruption or degradation of the organized nucleic acid systems directing adaptive enzyme synthesis. Bacteriophage are composed of nucleoprotein and they can infect and reproduce in irradiated cells of *Esch. coli*, although such cells are unable to form the adaptive enzyme β-galatosidase [19], and this may be construed to mean that irradiated cells can still synthesize

proteins provided the appropriate nucleic acid framework is made available.

By using materials enriched with radio-active phosphorus (P^{32}), several workers have measured the turnover rate of nucleic acid phosphate groups and the effect thereon of variations in the rate of protein synthesis. All the results reported so far are based on isotopic analyses of impure preparations of nucleic acid and must therefore be treated with caution since Davidson and his colleagues have shown that unequivocal results are obtained only if these substances are rigorously freed from contaminating materials before determining their P^{32} content [7]. Spiegelman and Kamen [39] have found that the fermentation of glucose by washed suspensions of yeast previously grown in the presence of inorganic phosphate enriched with P^{32} did not cause any decrease in the concentration of P^{32} in the nucleic acid fraction of the cells. However, if NH_4^+ was added to the system, protein synthesis and budding occurred and the P^{32} content of the nucleic acids decreased. A similar decrease took place during the adaptive formation of maltase. The proposal was therefore advanced that the energy in the phosphate bonds of the nucleic acid could be utilized in transfer reactions for the synthesis of peptide bonds.

From this brief survey it will be apparent that whilst there is some experimental evidence that nucleic acids mediate protein synthesis, our present conceptions of the cellular organization controlling and bringing about such syntheses are purely speculative (see [51] for a critical analysis of possible mechanisms for the synthesis of proteins).

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CHAPTER VIII

PROTEOLYTIC ENZYMES

PUTREFACTION of the remains of dead animals and plants in natural environments is due in part to autolysis, i.e. to disintegration of cellular components by the organism's own enzymes [27], and in part to the activities of the mixed population of micro-organisms which rapidly becomes established in such conditions. Because of their insolubility or molecular size, many cellular materials must be degraded to simpler substances before they can be utilized by micro-organisms as sources of carbon, nitrogen and energy. Degradation may be accomplished either by enzymes present in the cell wall of the micro-organism, in which case the organism must itself come into physical contact with the substrate (e.g. the digestion of cellulose by species of *Cytophaga*), or by extracellular enzymes produced by the micro-organism yet acting independently of the parent cell. One group of extracellular enzymes especially important in putrefaction is the proteases, enzymes which hydrolyse peptide bonds and thus make available the amino-acid constituents of proteins.

For several years bacteriologists have used physical manifestations of proteolytic activity in the classification and identification of micro-organisms, e.g. the liquefaction of gelatin or the clotting and digestion of milk. Moreover, the unwelcome effects of some pathogenic bacteria are now known to be due to extracellular toxins which possess enzymic activity against certain proteins in the susceptible host. Mainly because of the influence of industry and medicine, the proteolytic activity of bacteria has been studied almost exclusively in terms of natural substrates such as casein, collagen (or gelatin) and fibrin, and more from the viewpoint of the bacteriologist rather than the biochemist. On the other hand, there have been considerable advances in knowledge with regard to animal proteases, several of

which have been crystallized, and a summary of the more important results of this work provides a background against which the limited information concerning microbial proteases can be considered.

Hydrolysis of proteins and peptides by animal enzymes [36]

Until 1935 the animal proteases were classified according to molecular size of substrate and pH for optimum activity, i.e. the emphasis was on physical properties. Proteins were regarded as being the substrates of the proteinases (pepsin, chymotrypsin and trypsin), whilst peptides, substances with a relatively small number of peptide bonds, were the substrates of the peptidases (aminopolypeptidases, carboxypolypeptidases and dipeptidases). The introduction by Bergmann of a new and relatively simple method for the chemical synthesis of small peptides of known composition stimulated a detailed inquiry into the specificity of enzymes capable of hydrolysing peptide bonds. By using these synthetic substrates it was shown that the main factors affecting whether a proteolytic enzyme hydrolysed a peptide bond were, firstly, the nature of the amino-acids linked together by the bond, and secondly, the absence or presence of free amino or carboxyl groups in the vicinity of susceptible bonds. Bergmann proposed that the proteases be grouped into the endopeptidases and the exopeptidases according to whether they hydrolysed peptide bonds remote from or near to the ends of peptide chains in natural substrates. Endopeptidases were typified by pepsin, trypsin and chymotrypsin: the activity of pepsin and trypsin is inhibited by free amino groups near to susceptible bonds whilst chymotrypsin, and possibly trypsin as well, is inhibited by neighbouring carboxyl groups. The exopeptidases comprise the dipeptidases and the amino- and carboxy-polypeptidases. The latter two groups of enzymes attack peptide bonds adjacent to, and in some cases penultimate to, terminal amino-acid residues with free amino groups and free carboxyl groups respectively. Whereas both proteins and peptides can serve as substrates for the endopeptidases, the exopeptidases only attack peptides. The endopeptidases, unlike some of the

exopeptidases, cannot hydrolyse bonds involving D-amino-acids. Bergmann and Fruton's investigations appeared to show that the specificity of the endopeptidases with regard to the amino-acid composition of the bonds they attacked was relatively high, and this was particularly so in the case of pepsin. But as more peptides have been synthesized and tested [see 2], it has become clear that these enzymes are not so specific as the results of the earlier work might suggest. Though few of them have yet been purified, it is evident that there are a number of dipeptidases, amino- and carboxy-polypeptidases and it is possible that each one has different specificity requirements.

In addition to the extracellular enzymes, there are a number of intracellular animal proteases known as kathepsins, of which four types are known, analogous to pepsin, trypsin, amino-polypeptidases and carboxy-polypeptidases respectively as regards their specificity. Unlike the extracellular enzymes, the kathepsins are only active in the presence of an activator such as H_2S, cyanide, cysteine or glutathione, and in this respect they are very similar to some of the intracellular proteases of micro-organisms.

Great interest has been taken in the mammalian extracellular proteases known to be secreted in the form of an inactive precursor, a zymogen, the crystalline form of which is different from the corresponding active enzyme. Pepsinogen is converted to pepsin by treatment with acid or with pepsin itself, whilst trypsinogen is activated by enterokinase or by trypsin. Activation of the precursor may involve the removal of a small peptide (as with pepsinogen) or the opening of a small number of peptide bonds (chymotrypsinogen and trypsinogen).

There is at present conflicting evidence as to the manner in which proteins and peptides are hydrolysed by the endopeptidases to smaller units. The enzyme may attack all the susceptible peptide links in any one peptide chain simultaneously and thus release the component amino-acids and peptides concurrently (the 'all or none' hypothesis). Alternatively, it may attack the peptides in a random manner, hydrolysing only one bond at a time in any one peptide, until the

system eventually contains no more susceptible bonds. Tiselius, on the basis of an electrophoretic analysis of the reaction mixture obtained by the treatment of egg-white with pepsin, supported the former concept [39]. However, chromatographic analysis has revealed that though the system ultimately contained mostly tripeptides, a few dipeptides, no free amino-acids and still some undigested protein, in the initial stages of the reaction, deca- and higher peptides were present [31].

Proteases of micro-organisms

Only a small number of the proteases of micro-organisms have been purified and only one has been crystallized [14]. Apart from showing that they produce certain effects, e.g. the liquefaction of collagen and gelatin, there have been few attempts to express activity in terms of the hydrolysis of peptide bonds, and virtually none concerned with their specificity with respect to the bonds attacked [32]. Most of the experiments with synthetic substrates have been confined to the peptidases and no bacterial proteinase has been examined in such detail as mammalian pepsin. In addition to intracellular proteases, some micro-organisms also possess extracellular proteolytic enzymes and these enable proteins in the environment to be degraded at least to small peptides, if not to amino-acids, and the products are then absorbed and may be further degraded by intracellular enzymes.

The biological activity of extracellular substances produced by micro-organisms can be detected by incorporating the appropriate substrate in the culture medium or, more usually, by testing the activity of the culture filtrate. The test may be applied directly to the latter, or to a concentrate of the active principle prepared from the culture filtrate by evaporation *in vacuo*, by precipitation with ammonium sulphate or ethanol, by freeze drying, or by an adsorption technique. Enzymic activity of the experimental material is detected by incubating with the appropriate substrate, toxic activity by injecting the material into animals or by precipitation reactions with antisera, and antibiotic activity by

incorporating the material into culture media. The occurrence of enzymic activity in culture filtrates must not be taken as unequivocal evidence that the enzyme is extracellular, particularly if high activity is dependent on prolonged incubation of the cultures: in such circumstances it is natural to suspect that the organisms may have undergone partial autolysis and thus released intracellular enzymes into the medium. If a culture filtrate contains more than one protease, they may be separated from one another by the usual procedures employed in the purification of enzymes. Very active extracellular proteases are produced by species of *Proteus*, *Clostridium*, *Bacillus*, *Pseudomonas* and *Micrococcus*: less active enzymes are formed by the streptococci and staphylococci whilst the lactobacilli and Enterobacteriaceae (with the exception of *Proteus* spp.) apparently produce none.

Collagenase and gelatinase activity

The ability of certain bacteria to liquefy collagen and the material derived from it, gelatin, was soon discovered following the introduction of gelatin as a means of solidifying culture media [12]. One of the methods employed in the quantitative determination of gelatinase activity makes use of an Ostwald viscometer [13], the method being based on the assumption that changes in the viscosity of a solution of gelatin are an index of proteolytic activity. Such a procedure is indirect, and the observed changes in viscosity may not necessarily be due to the hydrolysis of peptide bonds. It is therefore desirable that viscometric methods should be compared with those that are based more directly on the results of proteolytic activity, and in particular on the expected appearance of free amino and carboxyl groups in the system. Suitable methods of this type employ the van Slyke apparatus for the determination of amino groups and the Sörensen titration procedure for carboxyl groups. Indeed, it is evident from the work of Gorini and Fromageot that changes in viscosity may bear no relation to the appearance of free amino groups (Fig. 8.1), but such a correlation has not often been attempted.

The most detailed studies of the proteolytic enzymes in bacterial culture filtrates are those of Maschmann [29], who isolated from the culture filtrates of the invasive clostridia (*Cl. histolyticum*, *Cl. welchii*, *Cl. septicum*) an enzyme that attacked gelatin and collagen but had no action on casein, peptone or egg albumin. Even filtrates from young cultures

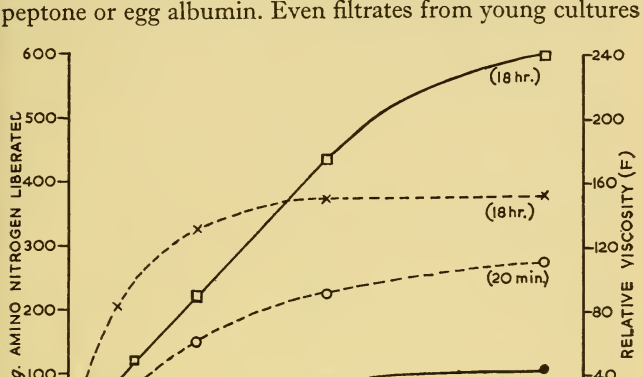


FIG. 8.1.—Hydrolysis of gelatin by extracellular proteinase of *Micrococcus lysodeikticus* as a function of enzyme concentration. Activity measured by appearance of amino nitrogen (solid line) and by changes in viscosity (broken line). Reaction times shown in parentheses. (From Gorini, L. and Fromageot, C. (1950), *Biochim. Biophys. Acta*, **5**, 524; Elsevier Publishing Co. Inc., New York and Amsterdam)

contained this specific gelatinase, and it was therefore regarded as being a truly extracellular enzyme. The invasive properties of certain clostridia can be ascribed to their ability to secrete this enzyme since Maschmann could not detect any specific gelatinase in filtrates from the non-invasive clostridia (*Cl. botulinum*, *Cl. tetani*), and later workers showed that the culture filtrates of the invasive clostridia digested the collagen supporting material of muscle whereas an enzyme such as trypsin liquefied the muscle fibrils and

left the connective tissue intact [34, 15]. The collagenase of *Cl. welchii* type A, also known as the kappa (κ) toxin, has been purified considerably [11]. Although on a weight basis this material was less toxic than the lecithinase (α-toxin) of *Cl. welchii*, it was ten to fifty times more toxic than the endotoxins of Gram-negative bacteria. A curious feature of the purified collagenase is that after exposure to mild alkali or to heat, it still attacked gelatin but not collagen [9]. This treatment may have altered the structure of the enzyme such that it can combine only with gelatin, and this may also be the reason why there is a change in the optimum pH for gelatinase activity. Another possible, though perhaps unlikely, explanation is that exposure to heat or alkali denatured the collagenase and at the same time activated a specific gelatinase precursor [9]. Some confusion exists in the naming of the enzymes which attack gelatin and collagen and it is sometimes assumed that the terms gelatinase and collagenase are synonymous. However, Evans and Wardlaw have evidence that though the formation of collagenase by various species of bacilli is always accompanied by gelatinase activity, some organisms, e.g. *B. subtilis*, produce a gelatinase and yet have no apparent action on collagen [16].

Other proteinases of the Clostridia

Apart from the specific gelatinase, Maschmann found three other types of proteolytic enzymes in various bacterial culture filtrates (Table 8.1). One type was active against proteins and peptones, and like the specific gelatinase, was found in young as well as old cultures and activity was unaffected by the presence of O_2: gelatin was also digested, but usually not as rapidly as casein. The two other types of proteases, one active against proteins, the other against peptides, appeared in the medium after the cultures had stopped growing, and it is therefore possible that at least in some instances they were intracellular enzymes released by autolysis. Their activity was depressed by O_2 and was only maximal in the presence of reducing substances (H_2S, cysteine or glutathione) whilst in addition, the peptidases also required divalent cations (Mg^{++}, Fe^{++} or Mn^{++}). The O_2-labile

proteinases were found in cultures of *Cl. welchii*, *Cl. histolyticum*, *Cl. botulinum* and *Cl. septicum*; peptidases were present in cultures of all the organisms named in Table 8.1. There may be strain differences with regard to the enzymes formed by a particular species. For example, the proteinase of *Cl. histolyticum* isolated by Maschmann was distinguished from that isolated by later workers in not being activated by cysteine. Further investigations revealed that some strains of *Cl. histolyticum* produced both of these proteinases [23].

TABLE 8.1

PROTEOLYTIC ENZYMES OF ORGANISMS STUDIED BY MASCHMANN

Organism	Specific gelatinase	Proteases, attacking casein and peptone, inhibited by normal serum	
	Enzyme stable in oxygen	Enzymes stable in oxygen	Enzymes labile in oxygen
Cl. botulinum	−	+	+
Cl. feseri	+	−	−
Cl. histolyticum	+	+	+
Cl. septicum	+	−	+
Cl. sporogenes	−	+	+
Cl. tetani	−	+	−
Cl. welchii	+	−	+
Ps. aeruginosa	−	+	−
Ps. fluorescens	−	+	−
Serratia marcescens	−	+	−

The one activated by Fe^{++} and thiol compounds attacked casein and clupein as well as gelatin, and liberated more carboxyl groups than amino groups, indicating that peptide bonds involving the amino group of proline had been hydrolysed [28]. The lambda (λ) toxin of *Cl. welchii*, purified by Bidwell, is a proteolytic enzyme capable of hydrolysing gelatin, casein, haemoglobin and hide powder, but it has no effect on native collagen [10]. It is strongly inhibited by cysteine, and, though there is no evidence that it is activated by Mg^{++} or Mn^{++}, by citrate.

Maschmann observed that the proteinases, but not the specific gelatinase, were inhibited by normal (i.e. not necessarily immune) sera. A more recent careful kinetic study of the proteolytic activity of several bacteria disproved the idea that this effect was due to the same substance that inhibits trypsin [13]. The trypsin inhibitor is found only in the albumin fraction of the serum proteins, whilst the labile antibacterial protease factor is in the globulin fraction: furthermore, the bacterial enzymes are not inhibited by the trypsin inhibitors present in soya bean, the pancreas and ovomucoid. The antibacterial serum factor inhibited all the bacterial proteases examined, even the specific gelatinases to a small extent. These investigations of Duthie and Lorenz also confirmed that the ability to clot milk is restricted to certain bacteria, and showed that although inhibited by the globulins of normal sera, the rate of clotting bore no relationship to the protease activity of the culture filtrates.

Factors affecting the formation and activity of extracellular proteinases

The composition of the growth medium profoundly affects the degree of proteinase activity that is ultimately detectable in the culture filtrate. Three factors appear to be of special significance, (i) inorganic ions, (ii) fermentable carbohydrate and (iii) the organism's source of nitrogen. A previous observation [30] that protease production by a species of *Proteus* depended on the presence of Ca^{++} and Mg^{++} was investigated in more detail by Hanes, using the organisms *Bacillus subtilis*, *B. mesentericus*, *Pr. vulgaris*, *Ps. fluorescens* and *Ps. aeruginosa* [22]. The addition of Ca^{++} had little effect on growth, but markedly increased the production of gelatinase, whilst Mg^{++}, although increasing growth, caused no increase in the gelatinase activity of the cultures. The recent work of Gorini has served to emphasize the important role of cations such as Ca^{++} [17]. Optimal extracellular protease activity of *Micrococcus lysodeikticus*, *B. megatherium*, *Proteus*, *Ps. pyocyanea* and *B. mesentericus* was dependent on growth at a low temperature (26° C.) in

well-aerated media in the presence of Ca^{++}. The amount of glucose added to the medium had to be such that the pH at the end of growth had not fallen below pH 7. Gorini contends that as well as activating the proteases, Ca^{++} also has a stabilizing influence and that in the absence of Ca^{++} these enzymes are inactivated as fast as they appear in the medium [19]. This view is opposed to that of previous workers who believed that Ca^{++} stimulated the actual *production* of the enzymes. When proteins and polypeptides were used as a source of nitrogen, the growth of the culture was dependent on its proteolytic activity, and in such conditions Ca^{++} was indispensable for growth [18]. Sodium fluoride and citrate, substances capable of combining with Ca^{++}, inhibited these proteases, and although Mg^{++} protected them against such inhibitors and from denaturation by heat, Mg^{++} could not replace Ca^{++} as the cationic activator [17]. There is some evidence that the gelatinase activity of cultures of *B. subtilis* is dependent on the Mn^{++} content of the medium [38].

Although several workers have regularly demonstrated activity in filtrates from cultures grown in media containing glucose [30], other workers have reported that the presence of fermentable carbohydrate inhibits the formation of proteolytic enzymes [3]. Such effects are probably to be attributed to the acidic products of carbohydrate catabolism causing the pH of the medium to fall to a value which does not favour the formation of proteases. In buffered media, or in those where the pH does not become acid [cf. 17, 19] the presence of fermentable carbohydrate has no effect [3].

Several attempts have been made to ascertain whether the growth of proteolytic bacteria is supported by pure native proteins, or only by those which have been denatured or partially degraded. Such bacteria failed to grow when subcultured into a medium containing inorganic salts and a pure protein as a source of carbon and nitrogen [1]. This might be explained on the basis that the synthesis and excretion of an extracellular enzyme involves the utilization of energy,

which in these experiments could only be derived from amino-acids after they have been made available by enzymic hydrolysis of the protein. Even if any extracellular enzyme is carried over in the inoculum it is possible that it is diluted in the subculture to such an extent that its activity is no longer significant. If a small amount of peptone, presumably containing some small peptides or amino-acids, was added to the medium, certain proteolytic bacteria, e.g. *Pr. vulgaris*, grew rapidly and crystalline egg albumin and serum proteins were then readily degraded and utilized [37]. Hence it appears that the formation of extracellular enzymes is dependent on the medium containing sources of carbon, nitrogen and energy that can be utilized immediately without having to be first broken down into smaller units by extracellular enzymes. In any event, proteoses and peptones are apparently not attacked by members of the Bacteriaceae or by *Staph. aureus* or *Strep. faecalis* [33].

Specific proteinases of the streptococci and staphylococci

The culture filtrates of some Lancefield group A streptococci contain an O_2-labile papain-like enzyme which apart from hydrolysing fibrin, casein, gelatin and benzoyl-L-arginamide, also attacked the M antigen, one of the antigens used in typing group A streptococci [14]. The M antigen is usually absent in those strains capable of producing this enzyme, though it may be present if the cultures are grown at a low temperature (22° C.). After passage through mice, formation of the enzyme ceased and the organisms became more virulent and possessed the M antigen. The latter two effects are not directly related, since the M antigen is also present in some avirulent strains. Of some clinical importance is the fibrinolytic activity of haemolytic streptococci (groups A and C), staphylococci and gas-gangrene clostridia. These bacteria produce an enzyme which converts a precursor (plasminogen) in the globulin fraction of human sera into an active enzyme (plasmin) which digests the fibrin of clotted blood. The mode of action of the bacterial enzyme, named streptokinase in the case of streptococci, is comparable with the activation of chymotrypsinogen by trypsin.

Infection with *Strep. haemolyticus* soon results in the formation of an antibody which completely antagonizes streptokinase.

Peptidases of bacteria, aspergilli and yeast [4, 25, 36]

A number of observations have provided evidence for the occurrence of peptidases in micro-organisms similar to those in animals and plants, and in general their activity has been studied using simple substrates, di- and tri-peptides of glycine, alanine and leucine. Many, but not all, of these enzymes are activated either by thiol compounds or by divalent cations: some require both types of activator (Table 8.2). The peptidases of the clostridia exhibit poor activity

TABLE 8.2

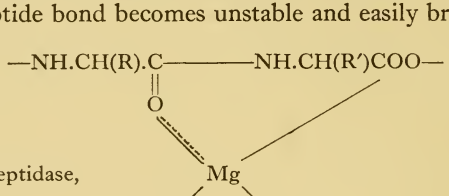
ACTIVATION OF MICROBIAL PEPTIDASES

The activators listed below are those which have been found to increase the activity of various preparations of peptidases from the organisms shown in the table. Which of these substances are the most effective activators for particular enzyme preparations depends on the peptidase concerned and sometimes on the substrate being tested.

Organism	Activators
Aspergillus parasiticus	Zn^{++}, cysteine
Bacillus megatherium	Zn^{++}, Mn^{++}, Fe^{++}, cysteine
Clostridium histolyticum	Mn^{++}, Fe^{++}, cysteine
Escherichia coli	Mg^{++}, Mn^{++}
Leuconostoc mesenteroides	Zn^{++}, Mn^{++}, Cd^{++}, Pb^{++}, cysteine
Phytomonas tumifaciens	Mg^{++}, Mn^{++}, cysteine
Proteus vulgaris	Mg^{++}, Mn^{++}
Pseudomonas fluorescens	Mg^{++}, Mn^{++}
Saccharomyces cerevisiae	Zn^{++}, Mn^{++}, Fe^{++}, Cl^{-}, Br^{-}, NO_3^{-}

except in the presence of cysteine together with a cation such as Fe^{++} or Mn^{++} (concentrations of the order 10^{-3} to 10^{-5} M.). For the hydrolysis of leucyl peptides, the best activator was Fe^{++}, for alanyl peptides, Mn^{++} was better [29]. Maschmann suggested that the active enzyme was formed by combination of the cation with the reduced form of the

'apoenzyme', and that the cation acted as a bridge joining the enzyme to the substrate. Smith [36] has proposed that the cation chelates with the substrate, and thus causes a redistribution of electrons with the result that the susceptible peptide bond becomes unstable and easily broken:



The co-ordination is believed to be between the —CO— group of the peptide bond and the terminal free carboxyl group for carboxypeptidases and the —NH— group and the free amino group for aminopeptidases. Examples are known in which co-ordination between cations and peptides is extremely specific, and this may explain why peptidases attacking different substrates are activated by different metal ions. The aerobic bacteria *Ps. aeruginosa*, *Ps. fluorescens* and *Serratia marcescens* contain a peptidase activated by Mg^{++} and comparable in specificity with the leucine aminopolypeptidase of animal tissues [5, 7]. Dipeptidases, or enzymes with activity against dipeptides, have been found in preparations from *Mycobacterium tuberculosis* [35], *Leuconostoc mesenteroides* [6], *Phytomonas tumifaciens* [5], *B. megatherium* [8], *Sac. cerevisiae* [20, 21] and *Aspergillus parasiticus* [7, 26]. A polypeptidase from *Sac. cerevisiae* and *Asp. parasiticus* was activated by Zn^{++}, and both the di- and poly-peptidase of yeast also required chloride ions [24] (Table 8.2). In general, the optimum pH for peptidase activity is in the range 8–9, but some peptidases of *Ln. mesenteroides*, propionibacteria and lactobacilli are most active at an acid pH (5·5–6·0), and they are not activated by divalent cations [5, 6].

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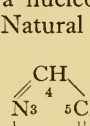
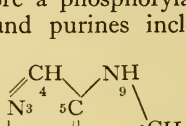
CHAPTER IX

NUCLEOTIDES AND NUCLEIC ACIDS

It requires but a short acquaintance with biochemistry to realize that the importance of nucleotides and nucleic acids is comparable with that of amino-acids and proteins. A nucleotide consists of a heterocyclic nitrogen compound, such as nicotinamide, a purine or a pyrimidine, joined to a sugar to which one or more orthophosphate groups are attached. The sugar is either a pentose (e.g. ribose) or a deoxypentose, and the dephosphorylated form of a nucleotide is known as a nucleoside. (The latter is really a N-glycoside and a nucleotide is therefore a phosphorylated N-glycoside.) Natural pyrimidines and purines include



pyrimidine,



and *purine,*

cytosine (4-amino-2-ketopyrimidine), uracil (2:4-diketopyrimidine), thymine (5-methyluracil), adenine (4-aminopurine) and guanine (2-amino-4-ketopurine).[1] The corresponding ribose nucleosides are known as cytidine, uridine, adenosine and guanosine, and the nucleotides as cytidylic, uridylic, adenylic and guanylic acid respectively. Plants and animals also contain 5-methylcytosine, but contrary to earlier reports, this pyrimidine is apparently not present in micro-organisms [51, 57].

Nucleotides are constituents of nucleic acids and also of the prosthetic groups and co-factors of many enzyme systems concerned with transfer reactions (e.g. ATP in energy transfer; DPN, TPN, FMN and FADN in hydrogen transfer;

[1] New system of numbering recommended by the Chemical Society (*J. Chem. Soc.*, 5064 (1952)).

Co.A in acyl group transfer). The recently discovered nucleotide, uridine-diphosphate-glucose, is a co-factor in the enzymic conversion of galactose-1-phosphate to glucose-1-phosphate [9]. With the exception of FMN, in which the glycone is ribitol, all these co-factor nucleotides contain β-ribose in the furanose form and phosphorylated on C′-5. In co-factors composed of two nucleotides the internucleotide bond is between the two 5-phosphate groups.

Nucleic acids are composed of large numbers of nucleotides and are consequently compounds of high molecular weight (of the order 5×10^5 to 1×10^6), but although the individual units are relatively simple, the determination of the detailed structure of a nucleic acid entails the solving of problems comparable in difficulty with those encountered in the elucidation of the structure of a protein (cf. p. 106). Depending on whether the component nucleotides contain either ribose or 2-deoxyribose, nucleic acids have been divided into two types, the ribose nucleic acids (RNA) and the deoxyribose nucleic acids (DNA). In only three instances has a derivative of ribose or 2-deoxyribose been isolated and characterized, and in the absence of such evidence some workers [14] prefer the terms pentose nucleic acid (PNA) and deoxypentose nucleic acid (DPNA). Adenine, guanine and cytosine are constituents of all the known nucleic acids, and in addition a PNA contains uracil, whereas a DPNA contains thymine. In consequence of their large numbers of phosphate radicals, nucleic acids are highly acidic and readily form salt-like compounds with bases. However, at least in bacteria, the proteins associated with nucleic acids in nucleoproteins are not necessarily of the basic protamine or histone type [15].

Structure of nucleotides and nucleic acids

Only in a few instances is there adequate proof of the structure of the component nucleotides of nucleic acids, but by analogy it is assumed that they all follow the same general pattern. The sugar is present as the β-isomer and in the furanose form, with a glycosidic linkage between the reducing group (C′-1) and N-7 of the purines and N-1 of the

pyrimidines. Position C′-3 is phosphorylated in the deoxypentose nucleotides, and though it is probably the same in the pentose nucleotides, position C′-2 is a possible alternative. Since hydrolysis in certain conditions yielded four types of nucleotides, apparently in equimolecular proportions, all nucleic acids were at first thought to be polymers of units each of which contained the four nucleotides arranged in a straight chain or in a cyclic tetrad. An alternative suggestion was that the overall equivalent proportions did not necessarily imply such a regular arrangement but were due simply to a statistical mean. Such theories required that the ratio of purine-N to pyrimidine-N should be 2:1, but with the accumulation of precise quantitative data came the realization that few nucleic acids contained the four nucleotides, or even purines and pyrimidines, in equimolecular proportions. For example, three types of DPNA have been isolated; in the one found in animals, yeast and most bacteria, adenine and thymine predominate (AT type), in another, found in only a few bacteria, guanine and cytosine predominate (GC type), whereas in the third type isolated from strains of *Esch. coli*, the bases are in equimolecular proportions [13].

Although discovered over eighty years ago, detailed investigations of structure have been confined to only two nucleic acids, a RNA from yeast and a DRNA from the thymus, and of these most is known about the former. Markham and Smith [38] believe that yeast RNA is a mixture of comparatively short, straight chains of nucleotides in which the internucleotide linkage is between the phosphate at C′-3 (or C′-2) of one nucleotide and the hydroxyl group at C′-5 in the adjacent nucleotide. They have also presented evidence that some of the chains terminate in cyclic nucleotides, i.e. nucleotides in which the phosphate group forms a bridge between C′-2 and C′-3. By using ion exchange resins, previous workers had shown that each of the four nucleotides in alkaline hydrolysates of yeast RNA could be separated into two isomers, the 'a' and 'b' nucleotides, which were regarded as being the nucleoside 2′- and 3′-phosphates. It is now evident that only one of these isomers occurs naturally, and that the other is formed by

the hydrolysis of cyclic nucleotides which are either present initially in the nucleic acid or are produced during the hydrolysis procedure [38]. The products obtained by the digestion of RNA with ribonuclease are mainly mono- and di-nucleotides together with some polynucleotide material which, because it would not dialyse, was at first thought to be the 'core' of the nucleic acid and to be of high molecular weight. Markham and Smith have now shown that the 'core' consists of relatively small polynucleotides whose dialysis depends on the concentration of salt in the system [38].

Estimation of nucleic acids and their components

The procedures for estimating total nucleic acid are based on either ultraviolet spectrophotometry or on determinations of orthophosphate or sugar (pentose and deoxypentose). In the latter chemical methods the cells are first extracted with cold trichloracetic acid (TCA) and a fat solvent to remove acid-soluble compounds and phospholipoids. After the nucleic acids have been released from the nucleoproteins by heating the extracted cells with 5% TCA at 90° C. for 15 minutes, pentose is estimated colorimetrically by the orcinol method and deoxypentose by the Dische-diphenylamine method [48]. In the Schmidt and Thannhauser procedure [47] the extracted cells are treated with N.-KOH at 37° C. to hydrolyse the PNA to free nucleotides, and then the undegraded DPNA and protein are precipitated by making the hydrolysate normal to HCl. The DPNA and PNA content of the original material is calculated from the organic and inorganic phosphate in the hydrolysate and the organic phosphate in the DPNA fraction. This method assumes that the only cellular acid-insoluble phosphorus compounds are nucleic acids and phosphoproteins—an assumption now known to be invalid [42]. In both the sugar and phosphate methods, the results are expressed in terms of nucleic acid by using conversion factors based on thymus DNA and yeast RNA as standards.

A characteristic property of purines and pyrimidines is that they strongly absorb in the ultraviolet region of the spectrum, with a peak absorption in the region of 260 mμ.

It is therefore natural that spectrophotometric techniques should have been developed for the quantitative estimation of the free bases and the various substances in which they are constituents. The total nucleic acid content of organisms spread in a film on a slide can be determined by ultraviolet spectrophotometric microscopy [10], whilst for cell suspensions, there is a technically simpler procedure based on the use of a standard ultraviolet spectrophotometer [40]. By making various assumptions, an average value for the absorption coefficient of a typical nucleic acid can be deduced and thus the absorption measurements interpreted in terms of nucleic acid. The originators of these techniques are well aware that reliable results are only obtained if due account is taken of a large number of variables and that many of the basic premises may not be strictly valid [cf. 10, 40]. Nevertheless, even if the results are not entirely accurate in terms of precise quantitative values, they are extremely useful, particularly when the data are used in a comparative manner.

The identification and quantitative determination of the various bases and nucleotides present in a nucleic acid necessarily involves hydrolysis of the nucleic acid, and chemical hydrolysis without degradation of one or more of the nitrogenous bases presents some difficulty. Depending on the conditions employed, the products are nucleotides, nucleosides, the free bases or mixtures of these substances. Thus subjecting a PNA to mildly acidic conditions, e.g. N.-HCl at 100° C. for 1 hour, liberates the purines in the free state, but the pyrimidines still remain in nucleotide combination from which they are freed by more vigorous hydrolysis, e.g. formic acid at 175° C. [see 25]. On the other hand, alkaline hydrolysis of a PNA yields a mixture of the four nucleotides. The deoxypentose nucleic acids behave differently on chemical hydrolysis, and degradation to nucleosides and nucleotides is best achieved by the use of enzymes. In the past, separation of the end-products obtained by hydrolysis or enzymic digestion of nucleic acid was accomplished by precipitation as the phosphotungstate or as the uranium or silver salt, or by simply adjusting the pH of the system. More recently, ionophoresis on paper [38]

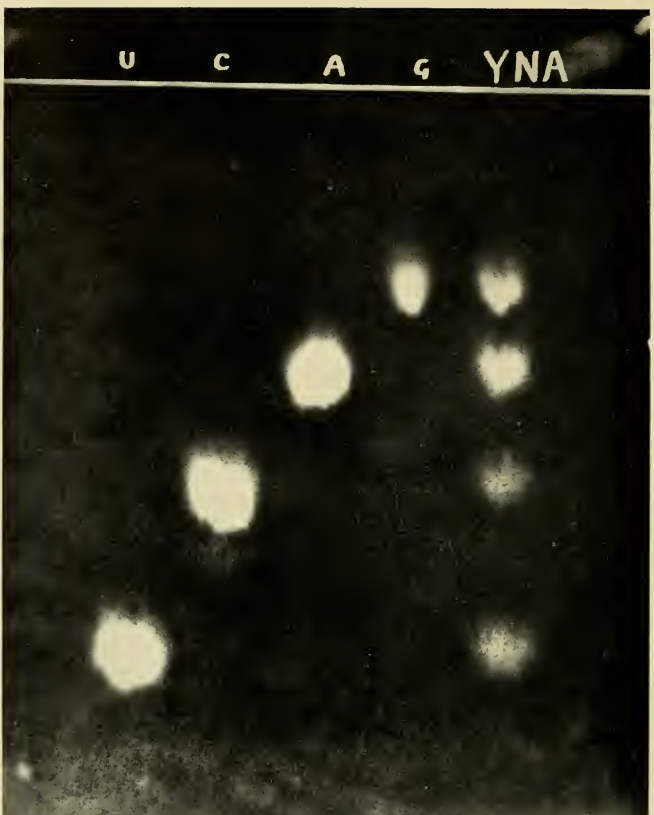


PLATE II.—Separation and identification of the purines and pyrimidines in yeast ribonucleic acid (YNA): YNA hydrolysed for 2 hr. in 70 %$HClO_4$ at 100° C.: solvent system isopropanol (65% v/v) and 2N-HCl (final concentration): G, A, C, and U pure samples of guanine, adenine, cytosine and uracil. Photograph obtained by placing chromatogram over reflex document paper and exposing to ultraviolet light. ST. denotes starting line

and chromatography, both on paper [11] and on ion exchange resins [18], have provided more convenient and precise techniques for the separation, preparation [19] and identification of nucleotides, nucleosides and the bases. When a paper chromatogram is exposed to light of wavelength 260 mμ, the areas occupied by purines, pyrimidines, nucleosides or nucleotides appear as dark spots on a light blue fluorescent background. A permanent photographic record can be obtained by placing the chromatogram over reflex copying paper [37], and the areas in the chromatogram containing compounds absorbing ultraviolet light will appear as white spots on a dark background in the developed photograph (Plate II). The appropriate areas of the chromatogram are then cut out, the compounds eluted and estimated spectrophotometrically.

Microbial nucleic acids

Micro-organisms, particularly bacteria, are richer in nucleic acid than most of the cells of other organisms, and Belozersky has calculated that 15–30% of the dry weight of bacteria is nucleic acid and 50–80% is nucleoprotein [4]. Prior to isolating a nucleic acid, soluble nucleotides and phospholipoids are first removed by successively extracting the cells with cold TCA and a fat solvent. The residue is then treated with dilute solutions of an alkali, e.g. 0·2% NaOH or Na_2CO_3 in order to extract the nucleic acids, the details of the procedure varying according to the nucleic acid required. The isolation of microbial nucleoproteins, especially in an undegraded or 'native' state, presents many difficulties and, apart from low yields, it is doubtful if any of the present techniques are ideal. The most favoured method is to extract the cells with neutral solutions of NaCl at a concentration depending on the type of nucleic acid it is desired to isolate: even so, the deoxypentose nucleoproteins adhere strongly to the cell structure and are only removed with difficulty [4]. When an aqueous solution of a nucleoprotein is shaken with chloroform, the protein becomes denatured and collects at the interface, whilst the liberated nucleic acid remains in the water phase. The

sodium salt of the nucleic acid can then be precipitated by the addition of ethanol, or, alternatively, the free nucleic acid by the addition of acidified ethanol or glacial acetic acid.

The staining procedure introduced by Christian Gram to reveal the presence of bacteria in animal tissues was subsequently developed into an empirical technique for dividing bacteria into two groups. After the fixed organisms have been stained with a basic dye (crystal violet or methyl violet) at pH 7–8, they are treated with a mordant, usually I_2 in KI, and then washed with ethanol or acetone. If the stain is quickly removed, the organism is said to be Gram-negative; whereas if the dye remains, it is regarded as being Gram-positive. After the ethanol or acetone treatment, it is now common practice to counterstain with a red dye, with the result that in the final preparation Gram-positive organisms are stained blue whilst Gram-negative organisms are red. Though the mechanism of this staining reaction is still a matter of dispute [see 3, 41], retention of the basic dye by Gram-positive bacteria appears to be due to the presence of a Mg^{++}-PNA complex in the peripheral layers of the cells. The evidence for this belief rests on the observation that after treatment of the dead cells with ribonuclease—an enzyme depolymerizing PNA (p. 134)—or with a detergent such as bile salts, Gram-positive organisms stain as though they were Gram-negative. The action of the detergent is to liberate PNA from the cells and this material has been isolated by Henry and Stacey, who found that in the presence of Mg^{++} and a reducing substance, the isolated PNA, or indeed yeast RNA, would restore Gram-positive staining properties to the appropriate Gram-negative cytoskeletons [21], but not to truly Gram-negative organisms. Though the ratio of PNA:DPNA was reported to be 8:1 in Gram-positive as opposed to 1·3:1 in Gram-negative bacteria, these figures are disputed by Mitchell and Moyle who claim that the ratio is about 4:1 in all bacteria, irrespective of their staining properties [41]. Moreover, the presence of a peripheral layer containing PNA may not be a complete explanation of the structure responsible for the Gram-staining

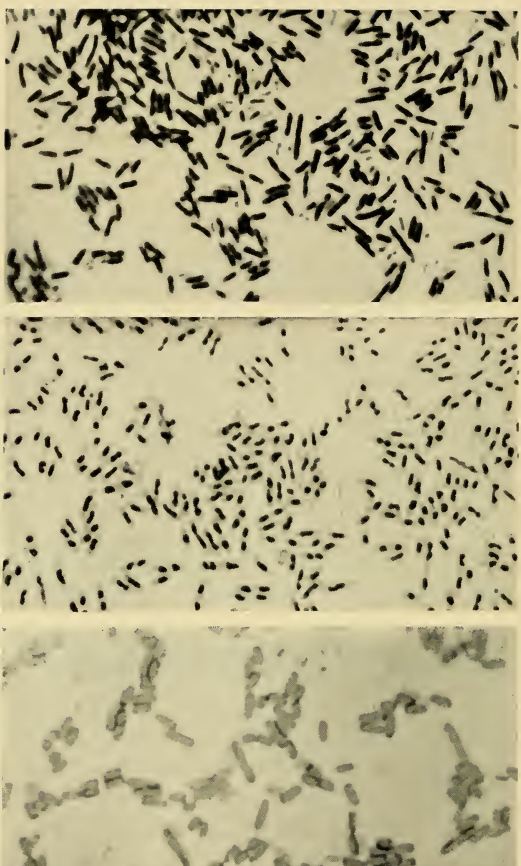


PLATE III.—Photographs of fixed cells of a colon bacillus stained by the Giemsa technique: 1, untreated cells; 2, fixed cells treated with ribonuclease prior to staining; 3, treated with ribonuclease and deoxyribonuclease. Ribonuclease removes cytoplasmic PNA responsible for the overall staining in 1 and enables the presence of chromatinic (nuclear) bodies to be shown (2). The latter are composed of DPNA and are not present in the cells treated with deoxyribonuclease (3)

reaction, since Gram-positive but not Gram-negative bacteria contain large amounts of the phosphates of various polyalcohols, in particular, glycerol. If a suspension of *Staph. aureus* is shaken with minute glass beads, the cells are disrupted, but the outer layer of the cell—the 'cell wall' or 'cell envelope'—remains intact, and it is with this that the major portion of the polyol phosphates is associated [42]. However, no direct evidence has yet been presented that these phosphates participate in the Gram-staining reaction. The recent work of Bartholomew and Mittwer has provided further support for the view that a layer immediately internal to the cell wall is the site of the Gram-staining reaction [3].

The nuclei of plant and animal cells are rich in nucleic acids, mostly of the DPNA type, and it is now generally accepted that transmission of hereditable characters is associated with these substances. Whether or not bacteria possess a nucleus has been the subject of endless and inconclusive discussion, but they do undoubtedly contain structures composed of DPNA and known as chromatinic bodies. The latter can be demonstrated in living cells by dark ground phase contrast microscopy [50] and in dead cells by the Feulgen or Giemsa staining technique. The Feulgen technique is based on the fact that after being subjected to acid hydrolysis, deoxypentose nucleic acids, but not nucleic acids of the pentose type, restore the colour of Schiff's reagent, consequently cellular structures composed of DPNA become stained magenta. Since any aldehyde is capable of giving a positive reaction, the results should not be accepted without confirmatory evidence. If an observed staining reaction is due to PNA or DPNA, it should no longer be given by material previously treated with the appropriate enzyme, ribonuclease or deoxyribonuclease (p. 134), and then washed (Plate III). Caution is also required in interpreting the results obtained in this manner since it may not be justifiable to assume (i) that the enzyme can penetrate the experimental material and thus come into contact with the substrate and (ii) that the enzyme preparation is specific in its activity.

Although the evidence is often indirect, there are good reasons for believing that in micro-organisms, as in the

more complex forms of life, the inheritance of specific characters is controlled by the deoxypentose nucleic acids, and in this respect a group of natural substances, known as transforming factors, are of particular interest. A transforming factor induces a susceptible cell to acquire a particular hereditable and characteristic property of the cell from which the factor emanates. Once a cell has been 'transformed', the acquired feature is transmitted through all subsequent generations. The most thoroughly investigated example of this phenomenon is provided by the pneumococci whose virulence is associated with the possession of a capsule of polysaccharide material. Differences in the composition of the latter have enabled the pneumococci to be grouped into more than thirty serologically distinct types. Griffith observed that living non-encapsulated avirulent type II pneumococci were changed into virulent encapsulated type III pneumococci by passage of the former together with heat killed cells of the latter through mice. Such transformations can be brought about *in vitro* in certain well-defined conditions and later Avery and his colleagues obtained convincing evidence that the agents responsible for the transformation of pneumococcal types were deoxypentose nucleic acids, each acid being specific for one type of transformation [see 1]. More recently, other transformations dealing with capsulation, resistance to penicillin and the ability to ferment particular sugars have been demonstrated with certain strains of *Haem. influenzae*, *Esch. coli*, *Shigella paradysenteriae*, *B. anthracis* and *Pr. vulgaris*; as in the pneumococci, the factors accomplishing these transformations appear to be deoxypentose nucleic acids [1]. Pentose nucleic acids have been implicated in the formation of stretolysin S, the O_2-stable haemolytic exotoxin of *Strep. haemolyticus* [7].

Enzymic degradation of nucleic acids [31]

The enzymic degradation of nucleic acids commences with disruption of the internucleotide linkages by ribonuclease or deoxyribonuclease, enzymes specific for the pentose and deoxypentose nucleic acids respectively: the nucleic acid is thus reduced to a mixture of mono-, di- and

a few oligo-nucleotides. Whilst the properties of the nucleases of mammals and yeast have been studied in some detail, the corresponding bacterial enzymes have received comparatively little attention. *Streptococcus haemolyticus* (Group A) secretes both types of nuclease into the medium [34, 8], and ribonuclease is particularly active during the initial stages of bacterial autolysis [cf. 23]. At least as far as ribonuclease is concerned, depolymerization is due to hydrolysis of the bond linking C′-5 of one nucleotide to the phosphate group attached to C′-3 (or C′-2) of an adjacent pyrimidine nucleotide. Analogous linkages between a pyrimidine and a purine nucleotide or between two purine nucleotides are stable to ribonuclease, and the enzyme may therefore be regarded as being a highly specific phosphodiesterase [38]. Nuclease activity can be determined by using a solution of the appropriate nucleic acid and following (i) changes in viscosity, (ii) the appearance of acid-soluble phosphorus compounds, (iii) the appearance of acidic groups or (iv) the decrease in the absorption of ultraviolet light of wavelength 300 mμ.

After nucleotides have been dephosphorylated by apparently non-specific phosphatases, the resultant nucleosides may be attacked in one of three ways [28]. One type of nucleosidase catalyses their phosphorolytic decomposition to the free base and a pentose phosphate by transferring the sugar moiety to inorganic orthophosphate (reaction *a*). The phosphorolytic nucleosidases of *Esch. coli* exhibit specificity with regard to the nitrogenous base in that they attack either purine compounds or pyrimidine compounds, but not both, and yet no specificity is shown towards the sugar part of the substrates. *Lactobacillus helveticus* [33] and *Esch. coli* [28] possess another type of transferase, specific for deoxyribosides and utilizing not inorganic phosphate but a nitrogenous base as the acceptor (reaction *b*).

(*a*) uracil–riboside+H_3PO_4 ⇌ uracil+ribose–1-phosphate

(*b*) uracil–deoxyriboside+thymine ⇌
thymine–deoxyriboside+uracil

The hydrolytic decomposition of nucleosides to the free

base and sugar in the complete absence of phosphate and other nitrogenous bases has been observed with preparations from bakers' yeast [12] and *Lb. pentosus* [29].

The removal of amino groups from the nitrogenous bases is accomplished by highly specific deaminases, but apart from the cytosine deaminase of *Esch. coli* and yeast [cf. 14], the adenosine deaminase of *Neurospora* [35] and the cytidine deaminase of brewers' yeast and *Esch. coli* [53], little is known about the occurrence and properties of these enzymes in micro-organisms.

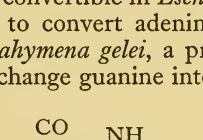
Oxidation and fermentation of purines and pyrimidines

Application of the enrichment culture technique has led to the isolation of organisms capable of utilizing purines and pyrimidines as sole sources of carbon, nitrogen and energy. Thus *Cl. cylindrosporum* ferments uric acid to NH_3, CO_2 and acetic acid, while *Cl. acidi urici*, in addition to uric acid, can also utilize xanthine, guanine and hypoxanthine [2]. Since neither of these species attacks allantoin, it has been suggested that they degrade uric acid by a route different from that found in animal tissues, and there is some evidence that glycine is an important intermediate in these fermentations. Unlike the clostridia, *Micrococcus aerogenes* attacks neither uric acid nor glycine, and lactic acid is the main acidic end-product in the fermentation of adenine or guanine [54]. On the other hand, *Micrococcus lactilyticus* is unable to utilize adenine, guanine or uric acid but ferments hypoxanthine and xanthine to H_2, CO_2, NH_3 and urea, together with propionic and acetic acids [55]. A number of aerobic bacteria (species of *Nocardia*, *Corynebacterium*, *Mycobacterium* and an unidentified soil organism) obtain carbon, nitrogen and energy by the oxidation of pyrimidines [30, 52]. Uracil and thymine are both oxidized to barbituric acid, which is then split into urea and malonic acid: the urea is subsequently decomposed by urease into CO_2 and NH_3, but the further steps in the metabolism of malonic acid are unknown. The oxidation of thymine proceeds by way of 5-methylbarbituric acid rather than by direct demethylation to uracil.

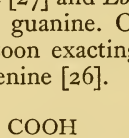
Synthesis of purines

Though the synthesis of the nitrogenous bases and the pentose sugars is here, for convenience, considered separately, such a division does not imply that the synthesis of nucleotides necessarily proceeds by the phosphorylation of a nucleoside formed by the joining together of the preformed base and the pentose sugar. There is some experimental evidence that glycosidation of a precursor precedes completion of the heterocyclic ring systems found in purines and pyrimidines. Moreover, the possibility must always be borne in mind that the routes of synthesis are not the same in different species. Experiments with substances labelled with isotopes have revealed that *Esch. coli* and yeast resemble mammals in that they synthesize purines and pyrimidines from relatively simple precursors, namely CO_2, NH_3, formate and glycine. In the synthesis of guanine by yeast, C-4 is derived from CO_2, C-2 and C-8 from formate or substances which give rise to formate (e.g. serine, the methyl group of methionine), C-6, C-5 and N-7 from the carboxyl, methene and amino-N of glycine respectively, and the remaining nitrogen atoms from NH_3 [20]. The utilization of other compounds for purine synthesis is indirect and involves their prior conversion to CO_2, NH_3, glycine or formate. Only one of the intermediates between these simple precursors and the completed purine is known. When *Esch. coli* is grown in the presence of sub-bacteriostatic concentrations of sulphanilamide or the folic acid analogue *N*-10-methylpteroylglutamic acid, an amine identified as 4-amino-imidazole-5-carboxamide (AIC) accumulates in the medium [49]. This substance only requires the addition of one carbon atom (corresponding to position 2) to complete the purine ring, and since it can be utilized by yeast, *Lb. arabinosus* and purine auxotrophs of *Esch. coli* and *Ophiostoma*, it is reasonable to suggest that it is a natural intermediate in purine synthesis. If glycine, a known precursor in purine synthesis, is added to the medium, production of the amine is increased [45], whereas it is reduced by methionine, especially if trace amounts of PAB are present, and also by vitamin B_{12}. Moreover, in the absence of purines,

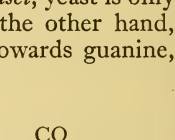
B_{12}, but not PAB or folic acid, enhanced the utilization of AIC by mutants of *Esch. coli* exacting towards purines [6]. These results are in accord with previous observations that the methyl groups of methionine serve as a source of one carbon units in intermediary metabolism and that they are related to formate, a substance known to be a precursor of purine carbon in position 2. Furthermore, B_{12} has already been implicated in the metabolism of labile methyl groups in mammals, and it is to be noted that this growth factor had no effect on the utilization of the formyl derivative of AIC, 4-formamino-imidazole-5-carboxamide, by the *Esch. coli* mutants. Incubation of AIC with *Esch. coli* resulted in the formation of a substance, possibly a pentoside, which was five times more effective than AIC itself in supporting the growth of the purine auxotrophs [5]. Though the routes by which the various purine bases are synthesized may be quite distinct, it is most likely that they have several steps in common, and it is possible that one is formed directly from another. The interconvertibility of the purines shows species variation; thus whilst guanine and adenine are freely interconvertible in *Esch. coli* [27] and *Lb. casei*, yeast is only able to convert adenine to guanine. On the other hand, *Tetrahymena gelei*, a protozoon exacting towards guanine, can change guanine into adenine [26].



Amino-imidazolecarboxamide



Aminofumaric acid



Orotic acid

Synthesis of pyrimidines

Although the pyrimidine ring system is also part of that present in purines, it is apparent that the synthesis of these two groups of nitrogenous bases proceeds by entirely independent routes. For example, in experiments with yeast, carbon from formate or glycine was incorporated into

guanine and adenine but not into uracil or cytosine. Furthermore, although carbon from isotopically labelled lactate was found in both types of compound, the resultant distribution of the isotope suggests that it entered the pyrimidines via oxaloacetate, and the purines via glycine [20]. Certain pyrimidine auxotrophs of *Neurospora* grow much better on uridine or cytidine than on the free bases, and this again suggests that the latter are not natural intermediates in nucleoside synthesis. Some of the mutants used orotic acid (uracil-6-carboxylic acid) to the same extent as uracil itself [32], whilst others, for which this replacement was not possible, accumulated orotic acid in the medium [39]. Though oxaloacetate, aminofumarate and aminofumaric acid diamide supported the growth of two of the mutants, they were only one tenth as effective as uracil. These and other observations led Houlahan and Mitchell to propose that in *Neurospora*, the biosynthetic sequence was: oxaloacetate $\rightarrow$ α-*N*-pentosylaminofumaric acid diamide $\rightarrow$ *A* $\rightarrow$ *B* $\rightarrow$ pyrimidine nucleoside: they also suggested that orotic acid is not a true intermediate but is related to the precursors *A* and *B*. In this connection it is interesting to note that a glycoside of orotic acid, probably the riboside, has been isolated from the mycelium of a uridine requiring mutant of *Neurospora*, and it is possible that this substance is a natural intermediate [43]. Incubation of *Sac. cerevisiae* with isotopically labelled orotic acid results in the appearance of the isotope in the uracil, but not the guanine, of the nucleic acids. Orotic acid has also been implicated in the synthesis of pyrimidines by streptococci, *Lb. casei* and *Lb. bulgaricus* [16]; the orotic acid requirements of the latter organism can be replaced by ureidosuccinic acid, a substance related to aminofumaric acid [56].

Synthesis of deoxyribose, ribose, nucleosides and nucleotides

A partially purified preparation of an aldolase obtained from *Esch. coli* has been shown to condense glyceraldehyde-3-phosphate with acetaldehyde and thus form deoxyribose-5-phosphate, which is converted by an isomerase, present in crude extracts, to deoxyribose-1-phosphate [44]. Whilst

ribose-5-phosphate may likewise be synthesized from phosphoglyceraldehyde and glycolaldehyde, *Esch. coli* and *Sac. cerevisiae* are known to be capable of performing the series of reactions, glucose → glucose-6-phosphate → 6-phosphogluconate → 3(2)-ketogluconic acid → ribulose-5-phosphate → ribose-5-phosphate → ribose-1-phosphate [22, 36]. In view of the reversible nature of the nucleosidases, it is reasonable to suggest that transfer reactions between the pentose-1-phosphates and purines or pyrimidines are a possible route of nucleoside synthesis. There is, however, no proof that this is the natural pathway and mention has already been made of some evidence, admittedly indirect, which indicates that glycosidation precedes completion of the heterocylic rings present in purines and pyrimidines. With regard to the phosphorylation of nucleosides to form nucleotides, all of our present knowledge concerns nucleotides known to be constituents of coenzymes rather than of nucleic acids. The following syntheses, catalysed by enzyme preparations obtained from yeast (*c*, *d*, *f*) and from liver (*e*, *g*) have been described [24, 46]:

(*c*) adenosine+ATP → adenosine-5-phosphate+ADP
(*d*) riboflavin+ATP → FMN+ADP
(*e*) nicotinamide-riboside+ATP → nicotinamide-riboside-5-phosphate+ADP
(*f*) FMN+ATP → FADN+pyrophosphate
(*g*) Nicotinamide-riboside-5-phosphate+ATP → DPN+pyrophosphate

Effect of bacteriophage on nucleic acid metabolism of host cell [17]

All viruses consist essentially of nucleic acids and proteins, and, in addition, the more complex types infecting animals also contain fatty material. Deoxypentose nucleic acids and PNA are found respectively in bacteriophages and plant viruses, and although most animal viruses contain only one type of nucleic acid, a few, e.g. the influenza virus, appear to contain both DPNA and PNA. When purified fully infective preparations of various viruses have been examined for the presence of known enzymes, no activity

has been found except in those of the more complex animal viruses. Interest has therefore centred around the mechanism whereby a virus assumes control of the metabolic activities of the host cell and reorganizes them for the synthesis of identical virus particles. The virus-host-cell system investigated in most detail is that concerned with the infection of *Esch. coli* B with a phage designated as the T phage, of which seven types are known. Electron microscopy has revealed that T1 and T5 are more or less spherical, whereas the other five T phages are club-shaped and have a distinct head, containing most of the DPNA, and a tail. Phage reproduction involves three phases: (i) adsorption on to and invasion of the susceptible cell; (ii) multiplication in the host cell, and (iii) liberation from the host cell, a process usually accompanied by lysis, though liberation and lysis are not always coincident. Unless stated to the contrary, the following description applies to phage T2 and the details are not necessarily the same for other viruses.

The adsorption of T2 by *Esch. coli* B is a rapid and reversible process dependent on the presence of tryptophan in the medium. It is soon followed by an irreversible process in which part of the virus, mainly DPNA and a little protein, enters the cell, an event which immediately results in a marked disturbance of the host cell's nucleic acid metabolism, the first visible signs being degradation of the chromatinic bodies. The inability of virus-infected cells to grow and divide is presumably an outward expression of this destruction of nuclear material. Nucleic acid synthesis is at first completely halted, but after a little while the synthesis of purines and pyrimidines commences and is soon followed by the formation of DPNA. Pentose nucleic acid synthesis is completely suppressed in *Esch. coli* B infected with T2, but in other virus-host-cell systems, the synthesis of both types of nucleic acid may take place. The amount of DPNA synthesized is equivalent to the total amount of both types of nucleic acid synthesized by uninfected cells, and this indicates that in the infected cells the units normally incorporated into PNA are being diverted to the synthesis of DPNA. In attempting to discover the reason

for this diversion Cohen has found that infection with phage causes a marked reduction in the ability of the cell to form ribose-5-phosphate from glucose-6-phosphate (p. 140), and this presumably may explain why such cells are unable to synthesize RNA. The synthesis of deoxyribose-5-phosphate from glucose-6-phosphate is unimpaired and consequently deoxypentose-nucleotide synthesis is unaffected.

Infection with phage has no visible effect on protein synthesis in the host cell, and although the amount of protein synthesized is in excess of that found in the number of virus particles eventually liberated, it is not yet known whether all the excess is viral protein. The origin of the various substances present in the phage has been determined by using cells whose cellular constituents have been enriched with isotopes and also by suspending the infected cells in media containing the appropriate compounds labelled with isotopes. As far as T2 is concerned, no more than 25% of the phosphorus, protein nitrogen, pyrimidines and purines in the liberated phage is derived from substances present in the host cell at the time of infection. How the virus particle gains control of the anabolic activities of the host cell is still unknown. It is however clear that the term 'self-duplicating particle' should not be used indiscriminately since, for example, the synthesis of protein and nucleic acid in infected cells proceeds at a linear rate, whereas if the system were self-duplicating, one might expect it to be autocatalytic. Moreover, there is evidence that the virus is radically changed during invasion of the host cell, since much of the phosphorus and some of the protein nitrogen of the infecting phage particle soon appears in the medium, and not for an appreciable time after invasion is it possible to isolate infective virus from the host cell. The assembling of the various units into completed virus appears to occur only a short time prior to liberation. In the T2 system, the first recognizable structures to be found in the infected cells are the collapsed heads of the phage containing only a little DPNA, the rest of the latter and the tails being added later.

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CHAPTER X

MODE OF ACTION OF CHEMOTHERAPEUTIC AGENTS

In recent years, the dramatic success of the sulphonamides and antibiotics in the treatment of bacterial and viral infections of man has focused much attention on the mode of action of these substances and the factors contributing to their activity. Substances interfering with the continued normal existence of an organism may owe their properties to (i) denaturation of proteins, (ii) disruption of cellular membranes and in consequence the leakage of essential metabolites, or (iii) more specifically, inhibition of particular enzymes. Substances bringing about the first two of these effects are of little chemotherapeutic importance, because of their adverse effect on the host as well as the pathogen. The key to successful chemotherapy is contained in the phrase *selective toxicity*, i.e. the drug is more toxic to the pathogen than to the host. If a chemotherapeutic agent acts by inhibiting an enzymic system of fundamental importance to the pathogen, then this system must be absent from the host, or, if present, it is for some reason less susceptible to the drug, or not essential. (For a detailed exposition of the principles of chemotherapy and selective toxicity, references 31 and 1 are recommended.) One of the aims of contemporary research is to establish a rational basis for chemotherapy, and with this in mind, the purpose of the following paragraphs is to survey very briefly the observed effects of the sulphonamides and antibiotics on the metabolism of nitrogenous compounds.

It must be emphasized that great care is required in deciding whether the observed effect produced by a drug is due to direct inhibition of the reaction leading to that end result, or whether it is a secondary effect arising out of the inhibition of some other, perhaps unknown, reaction. Even if the drug inhibits cell-free preparations of an enzyme this

does not constitute proof that its mode of action against the intact organism is necessarily explicable in such terms. Before the latter can be attempted, precise and quantitative information is required dealing with the significance of that enzyme in the general economy of the cell (e.g. turnover numbers). Moreover, ideally the observed results should be produced by drug concentrations of the same order as those used therapeutically. But, since the concentration of cells in washed suspension experiments is often many times greater than those in growing cultures or infected animals, it has been argued that this proviso can be ignored [14]; furthermore, it is also feasible that the drug enters growing and dividing cells more readily than resting cells [10].

Sulphonamides

The first major contribution towards understanding the mode of action of the sulphonamides was made by Woods who prepared from yeast an aromatic carboxylic amine which competitively antagonized the action of sulphanilamide in preventing the growth of *Strep. haemolyticus*. Fildes' suggestion that a chemotherapeutic agent might function by virtue of its chemical structure being such that it was adsorbed on to an enzyme in place of the natural substrate led Woods to infer that the isolated material was *p*-aminobenzoic acid (PAB) and he showed that the latter did in fact antagonize the sulphonamides in a competitive manner [28]. In other words, the biological activity of the sulphonamides could be explained on the grounds that they were non-utilizable analogues of a natural metabolite, namely, PAB. Until that time the importance of PAB in intermediary metabolism had not been suspected, but evidence soon became available that PAB was a growth factor for certain organisms, and that a group of substances containing PAB —the folic acid factors required by certain other organisms —were of universal importance. The various folic acid factors differ in the number of glutamic acid radicals in the molecule, the degree of reduction of the pterin and the presence or absence of a formyl group attached to one of the nitrogen atoms of the pterin or PAB. For example, the

'folic acid' requirements (citrovorum factor or folinic acid) of *Leuconostoc citrovorum* are replaceable by a synthetic material, *N*-5-formyl-5, 6, 7, 8-tetrahydropteroylglutamic acid, but not by pteroylglutamic acid itself (synthetic folic acid). The experimental evidence favours the view that organisms utilize PAB solely for the synthesis of 'folic acid' but the detailed constitution of the natural coenzyme containing combined PAB is still unknown [29].

The role of PAB in intermediary metabolism was explored further by a comprehensive survey of the substances other than folic acid which were capable of antagonizing the growth inhibitory action of the sulphonamides. This procedure is based on the hypothesis that if PAB or a derivative functions catalytically in the synthesis of substances essential for growth, and if the sulphonamides act by preventing the normal functioning of PAB, then growth should be resumed if these substances are supplied exogenously. In a sense such cultures can be regarded as being deficient in PAB or folic acid, and the principles involved are the same as in the growth factor replacement technique in which an attempt is made to replace a growth factor either by simpler substances from which it can be synthesized, or by substances whose synthesis the growth factor is suspected to mediate. Apart from folic acid, the natural antagonists of the sulphonamides fall into three groups, (i) amino-acids, in particular methionine and serine, (ii) purines such as xanthine and (iii) thymine and thymidine [22, 27]. For example, the addition of methionine to the medium decreased the amount of PAB required to overcome the inhibitory effects of sulphanilamide on the growth of *Esch. coli*. The PAB requirement was further reduced if xanthine was also included and diminished still further, if the medium contained methionine, xanthine and serine. It was abolished altogether when thymine was added in addition to these three substances. Similarly with a PAB auxotroph of *Esch. coli* growth was possible in the absence of PAB, provided the medium contained methionine, a purine and thymine. Analogous experiments have been done with other organisms and the same three groups of substances

are also active in antagonizing the growth inhibitory properties of analogues of folic acid, e.g. *x*-methylfolic acid.

Previous work with animal tissues and *Neurospora* indicated that serine was synthesized by the addition of formate

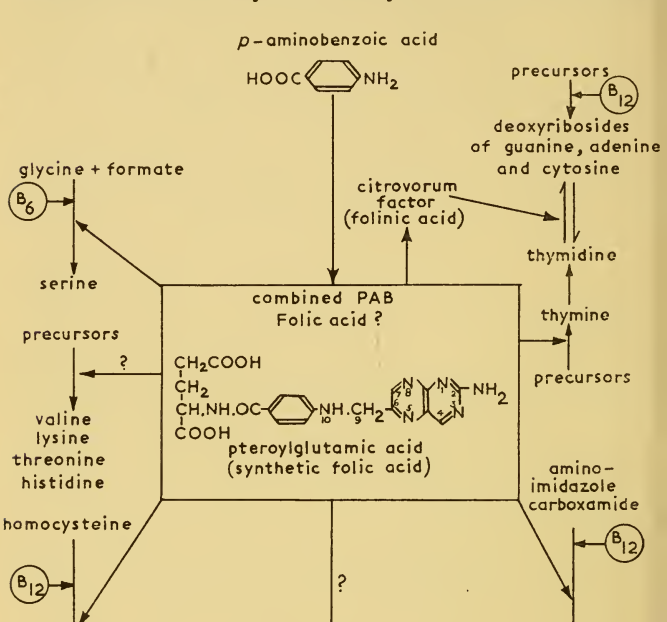

FIG. 10.1.—Role of *p*-aminobenzoic acid and vitamin B_{12} in the synthesis of amino-acids, purines and pyrimidines

to glycine, and since glycine, unlike serine, did not antagonize sulphanilamide, it was concluded that the latter was an inhibitor of this synthesis. Woods and his colleagues have now shown that though *Ln. mesenteroides* is exacting towards serine, growth occurred in the absence of the amino-acid

provided pyridoxin, PAB, CO_2 and glycine were available. Further evidence that pyridoxin as well as PAB participated in the synthesis of serine from glycine was obtained by using cells grown in the absence of these two growth factors in a medium containing all the other known growth factors together with amino-acids, purines and pyrimidines. Washed suspensions of these cells would synthesize serine only if they were provided with glycine, formate, glucose, pyridoxin and PAB (or folinic acid); synthesis was completely inhibited by sulphanilamide. Similar experiments have been done with *Strep. faecalis*, *Lb. bifidus* and *Sac. cerevisiae*. It appears that both PAB and vitamin B_{12} play a role in the synthesis of methionine, the most active amino-acid antagonist of the sulphonamides. A vitamin B_{12} auxotroph of *Esch. coli* grew in the absence of B_{12} if the medium contained methionine [4], while cells of a mutant requiring PAB and grown in the absence of this factor (i.e. in a medium containing amino-acids, purines and pyrimidines) only synthesized methionine from homocysteine in the presence of PAB and glucose. The simultaneous addition of B_{12} stimulated synthesis threefold, and recent work suggests that the methyl group used in the methylation is derived from serine. There is some evidence that the role of PAB in methionine synthesis cannot be explained solely on the basis that it is required for the synthesis of B_{12} and serine [29].

The connection between PAB and the synthesis of the group of substances now designated as vitamin B_{12} (the cobalamins) began with the observation that the anaemia produced by feeding an animal large amounts of sulphathiazole was like pernicious anaemia and could be relieved by large doses of synthetic folic acid or concentrates of substances isolated from the liver of normal animals. These liver substances were not of the folic acid type and functioned as growth factors for *Lb. leichmannii* and *Lb. lactis*. The key compound, vitamin B_{12}, active both as a growth factor and in the treatment of pernicious anaemia, has now been isolated in the crystalline state and is composed of 5:6-dimethylbenzimidazole-1-α-D-ribofuranoside-3-phosphate

combined with an unidentified organic molecule containing cobalt. Though the B_{12} requirements of a micro-organism cannot be replaced by any of the known folic acids, either synthetic or natural, they are replaceable by thymidine and also in most organisms, by the deoxyribosides of purines. This is to be contrasted with the fact that though thymidine replaces part of the PAB or folic acid requirements of an organism, all other deoxyribosides are inactive. Vitamin B_{12} contains an aromatic ring, yet it did not stimulate the growth of mutants with a multiple requirement for aromatic compounds. Davis therefore suggested that it was either derived from one of the aromatic substances required by such mutants or else it was synthesized by a totally different route or from an intermediate prior to the genetically blocked reaction. Vitamin B_{12} exerts a sparing effect on the PAB requirement of a PAB auxotroph of *Esch. coli*, and since the amount of vitamin B_{12} required is only one fiftieth of the amount of PAB, it is conceivable that the ring of PAB is used directly in the synthesis of B_{12} [3]. On the other hand, folic acid is known to be associated with the synthesis of purines, which, like B_{12}, contain an imidazole ring, and this may be the reason for the close relationship between these two co-factors. At the present time, the details of the relationship are but vaguely understood and have only been explored in one species, *Esch. coli.*

From these and other studies, it has become evident that the ultimate co-factor form of PAB participates in the methylation of homocysteine, the introduction of carbon into position 2 of the purine ring (pp. 137–8), and the synthesis of serine and thymine; in other words, this co-factor is concerned with the intermediary metabolism of one carbon units (cf. Co.A, the ultimate co-factor form of pantothenic acid, and its function in the metabolism of acyl units [20]). There is also reason to believe that B_{12} is involved in at least some of these reactions, e.g. the synthesis of methionine and purines, and it is to be noted that the presence of B_{12} increases the amount of sulphanilamide required to induce bacteriostasis of *Esch. coli* growing in the presence of one or more of the sulphonamide antagonists methionine,

xanthine, serine and thymine [21]. Other effects of the sulphonamides have been reported, but whether they are also the outcome of interference with reactions involving PAB is not always known, e.g. sulphathiazole appears to interfere with protein synthesis in *Staph. aureus*, but since protein synthesis involves a complex metabolic sequence of reactions, it is not possible to deduce whether this is a direct effect or simply due to disturbances in the synthesis of amino-acids or nucleotides [5]. It has been shown that the even- but not the odd-numbered T phages are unable to multiply in *Esch. coli* growing in media containing sulphanilamide together with methionine, serine, xanthine and thymidine [19]. The results of these and other experiments require to be interpreted with care, e.g. Pfiffner and his co-workers have isolated from bacteria compounds of the vitamin B_{12} group which contain adenine instead of dimethylbenzimidazole, and Davis later found that these substances (pseudo-B_{12}) replaced B_{12} in all respects for *Esch. coli* mutants requiring this vitamin or methionine.

Antibiotics

Though the precise details are still unknown, it is highly probable that the sulphonamides owe their activity to the fact that they are metabolic analogues. On the other hand, no such simple hypothesis is available to explain the activity of any of the antibiotics. There is no conclusive evidence that penicillin inhibits enzyme systems concerned in respiration or fermentation, and it is therefore unlikely that any of the results described below are attributable to direct interference with energy metabolism. By way of contrast, aureomycin resembles 2:4-dinitrophenol and sodium azide in that it may act as an uncoupling agent and prevent the production of energy-rich phosphate groups [15]. If penicillin (0·1–10 Oxford Units/ml.) was added to growing cultures of *Staph. aureus*, then within an hour of contact with the antibiotic the ability of the organism to accumulate amino-acids and synthesize protein progressively declined [8]. Penicillin had no effect on the uptake of glutamic acid and lysine by washed suspensions of normal cells, hence it

would appear that this antibiotic prevents the synthesis of the systems responsible for the absorption of amino-acids and does not affect the functioning of those systems once they have been established. When washed suspensions of cells grown for a short time with penicillin were incubated with glucose and glutamic acid, little of the latter accumulated in the cells, though extracellular peptides of glutamic acid appeared in the system [7]. These results are analogous to those of Hotchkiss, who used a different species of staphylococcus and different experimental conditions. Washed suspensions incubated with a mixture of various amino-acids and glucose synthesized protein, but in the presence of penicillin there was no increase in cellular combined amino-acids though the number of free amino-groups in the medium decreased. As in Gale's experiments, the latter was correlated with the appearance of extracellular peptides, and Hotchkiss suggested that penicillin inhibited protein synthesis and that these peptides were either intermediates in this process or were derived from them [13]. Nevertheless, it is difficult to believe that penicillin interferes directly with the synthesis of all proteins since it has no effect on the formation of adaptive enzymes, a process now regarded as being associated with the synthesis of new protein. Streptomycin, aureomycin, chloramphenicol and terramycin inhibit adaptive enzyme formation [11], an effect which is possibly the outcome of interference with energy metabolism.

By examining a number of strains of *Staph. aureus*, Gale found that increased resistance to penicillin could be correlated with a decline in ability to accumulate glutamic acid. It will be recalled that Gram-negative, unlike Gram-positive, organisms do not concentrate amino-acids in the cells (p. 82), and the most highly resistant variants of *Staph. aureus* obtained by successive subculture in increasing concentrations of penicillin were in fact Gram negative. Moreover, these organisms were no longer cocci but rod-shaped and had lost the ability to utilize certain sugars and grow anaerobically. Several workers have noted one or more of these effects (i.e. changes in morphology, Gram-staining

properties and ability to metabolize various substances, and also a preference for aerobic growth) with other species growing in the presence of penicillin. By studying whether the development of resistance is accompanied by overall changes in the metabolism of the organism, it may be possible to gain valuable information concerning the mode of action of the agent being considered, and furthermore, if resistance to other drugs is acquired simultaneously, i.e. cross resistance, it is conceivable that the biological effects of all these substances is explicable in the same terms [see 31]. Another as yet unexplained observation that penicillin interferes with amino-acid metabolism concerns an unidentified Gram-negative organism which when growing on L-leucylglycine in a mineral salt medium was relatively insensitive to penicillin. Leucine and glycine, either singly or together, also supported growth, but in the presence of uncombined glycine the organism was very sensitive to penicillin (1 to 10 units/ml.) [23].

The reports that the training of *Staph. aureus* to a high degree of resistance to penicillin resulted in a loss of Gram-positive staining properties could be taken to indicate that the biological effects of penicillin were the outcome of primary disturbances in nucleotide metabolism. In normal cultures of *Staph. aureus* the rate of cell growth appears to be controlled by the amount of pentose nucleic acid in the cells, and the cellular concentration of soluble nucleotides is inversely proportional to the rate of PNA synthesis [17]. If penicillin is added to a culture in the log phase of growth, then before there is any visible change in the growth-rate, the concentration of soluble nucleotides increases and the ratio of soluble nucleotides to total nucleic acid soon changes from 0·1 to 0·2 (Fig. 10.2). The percentage by weight of nucleic acid at first appears to increase, not because synthesis is stimulated but because there is a decrease in the rate of synthesis of some other substances (protein?) contributing to the dry weight of the cell. Though large amounts of nucleic acid are normally present in young cells, penicillin causes their concentration to fall rapidly to the low value characteristic of old cells in the stationary phase of growth,

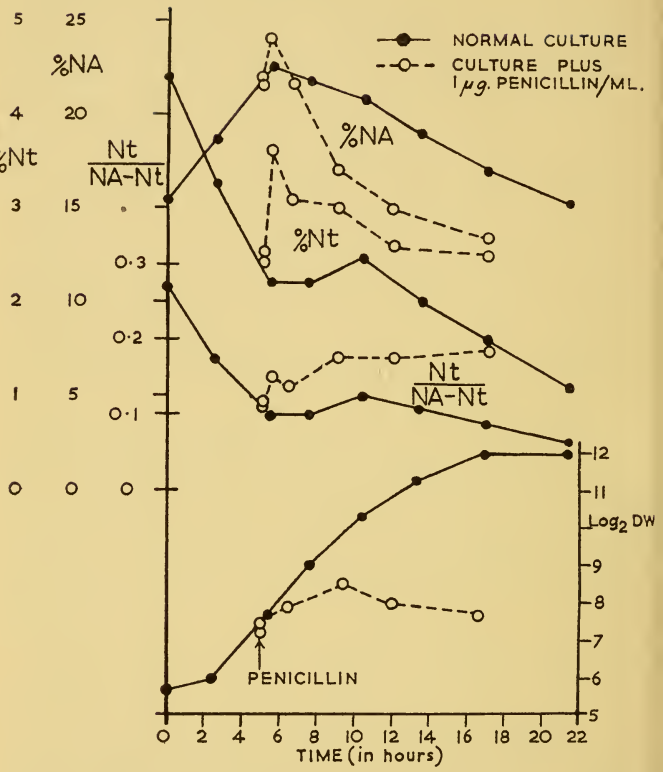

FIG. 10.2.—Effect of penicillin on nucleic acid metabolism of *Staph. aureus*. %NA=percentage by weight of total nucleotide, %Nt=percentage by weight of nucleotide extractable with 5% trichloracetic acid, NA-Nt=total nucleic acid, DW=dry weight of organism in μg./ml. [17]

and the ability to adsorb glutamic acid is not impaired until this process is taking place. From such results it may be deduced that penicillin interferes not with nucleotide synthesis but with their polymerization to nucleic acid. Park and Johnson have noted that the growth of *Staph. aureus* in the presence of penicillin (1 unit/ml.) leads to the accumulation of intracellular compounds containing uridine-5-pyrophosphate and an unidentified amino carboxylic sugar: a peptide of D-glutamic acid, D-valine and DL-lysine is a component of one of these compounds, whilst another contains L-alanine [18]. Synthesis of such nucleotides only occurs for as long as the cells are viable, and Park suggests that they are natural intermediates whose utilization is inhibited by penicillin. Maass and Johnson have shown that part of the penicillin absorbed by a cell is irreversibly bound within it, and they postulate that the antibiotic combines with and thus inhibits the natural functioning of a cellular constituent which is normally present in small amounts and controls the processes of cell division [16].

Penicillin is lethal to most Gram-positive organisms and is effective against only a few Gram-negative species, whereas streptomycin, chloramphenicol and aureomycin are active against a wide variety of organisms, and the two latter are also valuable in the treatment of diseases due to viruses and rickettsiae. Streptomycin had no effect on the accumulation of glutamic acid by *Staph. aureus*, but in concentrations markedly greater than those inhibiting growth, prevented protein synthesis. Aureomycin and chloramphenicol inhibited the absorption of glutamic acid and protein synthesis, the latter being especially sensitive [6, 7]. Of the many enzyme systems examined, only the diamine oxidase activity of whole cells of *Mycobacteria*, *Ps. aeruginosa* and *Staph. aureus* was inhibited by streptomycin, and there was some evidence that inhibition of this oxidase resulted in the cessation of growth [32]. Streptomycin contains basic guanidine groups and a possible explanation was that it was absorbed on to the enzyme in place of the natural basic substrate. However, cell-free preparations of the oxidase were but little affected by streptomycin, hence the observed

result is not due to direct inhibition of this enzyme [9]. The oxidase activity of whole cells of streptomycin resistant variants of *Myc. smegmatis* was very much less sensitive and like the mammalian enzyme, only inhibited by high concentrations [32]. Other experiments indicate that this antibiotic interferes with the entry of pyruvate into a terminal pathway responsible for its oxidation [12, 25], and that this pathway does not involve conversion to acetate and condensation of acetate with oxaloacetate to form citrate. Streptomycin-resistant strains of *Esch. coli* do not possess this pathway, and although it is also present in mammalian mitochondria, permeability barriers prevent streptomycin from having any effect [26]. Chloramphenicol inhibits esterases in the cell-free state, but had no effect on forty other enzymes examined [24]. There is some evidence that it interferes with the metabolism of aromatic amino-acids, e.g. with *Esch. coli*, the addition of phenylalanine, tyrosine or tryptophan overcame the growth inhibitory effects of *low* concentrations of chloramphenicol [30], and in *Esch. coli*, as in *Salm. typhosa*, it appears to prevent the conversion of anthranilic acid to indole [2].

Although the above account is very incomplete, it serves to illustrate that much has still to be discovered before a precise statement can be made concerning the mode of action of the sulphonamides and antibiotics. Nevertheless, apart from their potential value in the development of new chemotherapeutic agents, such studies have made and can make valuable contributions to the general pool of biochemical knowledge.

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