THE NITROGEN METABOLISM OF MICRO-ORGANISMS

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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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CONTENTS

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

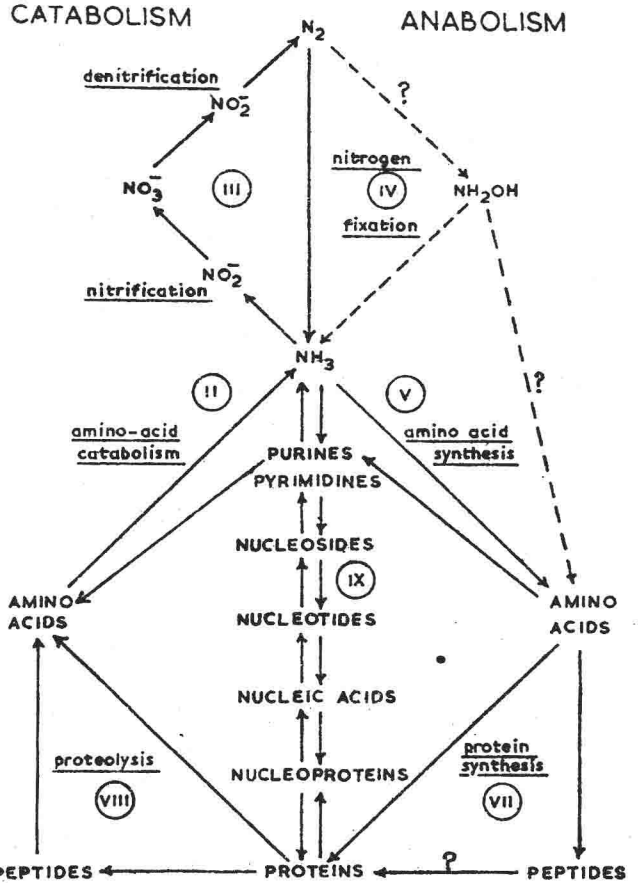

FIG. 1.1.—The Nitrogen Cycle. The roman numbers enclosed within circles denote the numbers of the chapters dealing with the various topics shown in the scheme

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.

BIBLIOGRAPHY

The following books are recommended for general reading and as reference books for detailed information concerning specific topics:

FOSTER, J. W. (1949), *Chemical Activities of Fungi*, Academic Press, U.S.A.

FRY, B. A. and PEEL, J. L. (editors), (1954), *Autotrophic Microorganisms, Soc. gen. Microbiol. Symp.*, **4**, Cambridge University Press, G.B.

GALE, E. F. (1949), *Chemical Activities of Bacteria*, University Tutorial Press, G.B.

LWOFF, A. (1951), *Biochemistry and Physiology of Protozoa*, Academic Press, U.S.A.

STEPHENSON, M. (1949), *Bacterial Metabolism*, Longmans Green, G.B.

SUMNER, J. B. and MYRBÄCK, K. (editors), (1950), *The Enzymes*, Academic Press, U.S.A.

WERKMAN, C. H. and WILSON, P. W. (editors), (1951), *Bacterial Physiology*, Academic Press, U.S.A.

REFERENCES

1. Albaum, H. G., Ogur, M. and Hirshfeld, A. (1950), *Arch. Biochem.*, **27**, 130
2. Barker, H. A. (1950), in *Phosphorus Metabolism*, **1**, 204 (Ed. McElroy, W. D. and Glass, B., Johns Hopkins Press, U.S.A.)
3. —— and Lipmann, F. (1949), *J. biol. Chem.*, **179**, 247
4. Dounce, A. L., Rothstein, A., Beyer, G. T., Meier, R. and Freer, R. M. (1948), *J. biol. Chem.*, **174**, 361
5. Gest, H. (1951), *Bact. Rev.*, **15**, 183
6. Hearon, J. Z. (1951), *Fed. Proc.*, **10**, 602
7. Hersey, D. F. and Ajl, S. J. (1951), *J. biol. Chem.*, **191**, 113
8. Kaplan, N. O. in *The Enzymes*, **2 (i)**, Chap. 45
9. LePage, G. A. and Umbreit, W. W. (1943), *J. biol. Chem.*, **147**, 263; **148**, 255
10. Lipmann, F. (1941), *Advances in Enzymology*, **1**, 99; (1946), **6**, 231; (1949), *Fed. Proc.*, **8**, 597
11. Lohmann, K. (1928), *Biochem. Z.*, **203**, 164

12. Lutwak-Mann, C. (1936), *Biochem. J.*, **30**, 1405
13. Mesrobeaunu, L. (1936), Thesis: Paris, *Contribution a l'étude des corps puriques de la cellule bacterienne*
14. Michaelis, L. in *The Enzymes*, **2 (i)**, Chap. 44
15. O'Kane, D. J. and Umbreit, W. W. (1942), *J. biol. Chem.*, **142**, 25
16. Racker, E. and Krimsky, I. (1952), *Nature*, **169**, 1043
17. Vishniac, W. and Ochoa, S. (1952), *J. biol. Chem.*, **195**, 75
18. Umbreit, W. W. (1947), *Bact. Rev.*, **11**, 157
19. *Bacterial Physiology*, Chaps. 11 and 19

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:

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