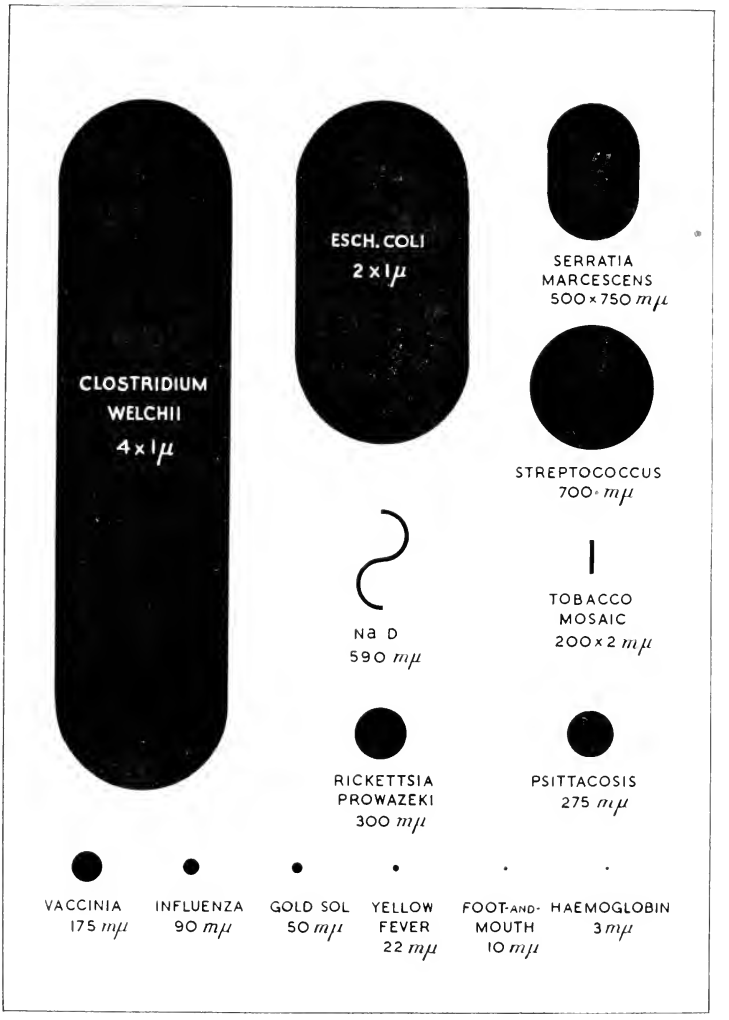
THE CHEMICAL ACTIVITIES OF BACTERIA



Relative Sizes of some Common Bacteria and Viruses.

THE CHEMICAL ACTIVITIES OF BACTERIA

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PREFACE TO THE THIRD EDITION

THE application of biochemical techniques to the study of bacteria has thrown considerable light on the mode of existence of these organisms and, in turn, such studies have assisted the development of our knowledge of the fundamental biochemistry of living cells. In this book I have attempted to produce an account of the chemical activities of bacteria which will be useful to students reading biochemistry, bacteriology, or chemistry, and which will provide an introduction to more advanced study. I hope also that the book will prove useful to research workers who require a concise but not detailed account of the background to present research in chemical microbiology. The treatment is elementary and in no sense comprehensive, and the bibliography has been limited, for the most part, to review articles and textbooks.

A comparatively new subject, such as this is, tends to develop rapidly and, although experimental facts must remain true, the accumulation of new facts inevitably alters the approach towards certain aspects of the subject. For example, during the last three or four years considerable advances have been made in our knowledge of synthetic systems and their control in the bacterial cell by "genes". I have attempted to incorporate some of these new ideas, and the facts on which they are based, in this third edition. In the course of making the changes I have found it desirable to alter the order of presentation of some of the material and to condense some sections in order that the book shall remain of approximately the same size.

I wish again to express my indebtedness to the late Dr Marjory Stephenson, F.R.S., for awakening and encouraging my interest in bacterial chemistry. I wish also to thank all those of my colleagues who have helped me to correct and avoid errors during the preparation of this book.

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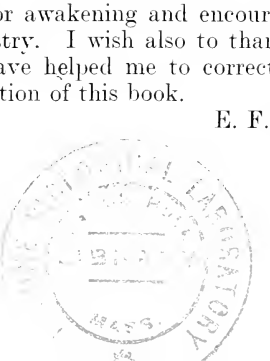
1951



CONTENTS

ADDENDUM

While this edition was in the press, Ochoa and co-workers have published a series of papers (*J. biol. Chem.*, 1950, **187**, 849 *et seq.*) dealing with the fixation of carbon dioxide by animal and bacterial cells. They have shown that CO_2 will combine with pyruvic acid in the presence of reduced coenzyme I (in bacteria) to form malic acid directly, the enzyme concerned being known at present as the "malic enzyme":—

$$CO_2 + CH_3.CO.COOH + CoE.H_2 = HOOC.CH_2.CHOH.COOH + CoE.$$

In the schemes put forward on pp. 135 and 137, the first stage in CO_2 fixation is shown as a carboxylation of pyruvic acid by reversal of oxalacetic decarboxylase. It has not been possible to demonstrate convincingly that the bacterial oxalacetic decarboxylase is reversible and the first stage in the fixation process should be represented as a direct formation of malic acid by the "malic enzyme" without the intermediate formation of oxalacetic acid.

In the "citric acid cycle" outlined in Fig. 12, citric acid is shown as a side-product not involved in the reactions of the actual cycle. Evidence has accumulated during the past year that citric acid itself is involved in the cycle. It is formed by the condensation of acetyl-phosphate and oxalacetic acid and then gives rise to *cis*-aconitic acid and the other substrates shown in the cycle. It is probable, in the light of the findings concerning the "malic enzyme," that the oxalacetic acid is formed via malic acid and not directly from CO_2 and pyruvic acid as shown in Fig. 12.

THE CHEMICAL ACTIVITIES OF BACTERIA

CHAPTER I

BACTERIA AS CHEMICAL AGENTS

The perceptible environment is composed of atoms, some existing separately, the great majority in constantly changing molecular combinations. The velocities of reaction between these molecular combinations vary over a very wide range and it is probable that only a minute fraction of the chemical changes occurring in the environment are perceived by man since the time for which he can make observations is so short. The chemist makes a study of these changes and endeavours to speed them up in the laboratory by such tricks as raising the temperature, adding strong acids or alkalis, introducing catalysts, etc. But if he studies the world outside his test-tubes and flasks, he soon becomes aware that biological material is able to carry out many types of chemical change with far greater ease and at considerably greater speed than he is able to achieve in his laboratory. In fact, one of the properties which differentiate between living and non-living material is this property of producing rapid and fundamental change in the chemical environment ("metabolism"). Living material is aggregated in organisms and we divide organisms for convenience into macro- and micro-organisms. The term "micro-organism" includes several subclasses such as the unicellular yeasts, protozoa, fungi, bacteria, etc., and it is amongst these organisms that we find the widest range and highest rates of metabolic activities.

As an index of the rate of metabolism we can take the rate of oxygen consumption which is usually measured as the c.mm. of oxygen taken up per hour by 1 mg. dry weight of cell material and is called the Q_{O_2} of the cells. If we compare the respiratory activities of various living

cells in this way, we get values for the Qo_2 of the following order:

Mammalian liver cells	$Qo_2 =$	2—5
Mammalian kidney cells		4—10
Yeast cells		50—100
Esch. coli	Bacteria	100—300
Acetobacter		*ca.* 1000
Azotobacter		*ca.* 3000

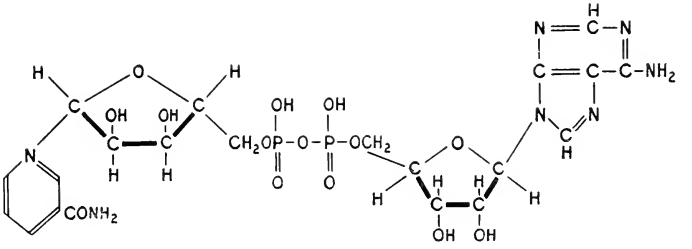
In this book we shall concern ourselves with bacteria only as, although it is by no means certain that these are the most active of the micro-organisms, they have been studied more intensively up to the present than the other types.

Bacteria have such a wide distribution that there are few places on or near the surface of the earth, in the waters of the earth, or in the air near the earth, which are free from them. They are found in hot mineral springs, in Arctic snows, in stagnant salt lakes, in oil-saturated soil around oil-wells, in the acid effluents from gas-works, etc. The only places free from bacteria are those in which a sterilising influence is at work; where heat, sunlight, or caustic chemicals render life impossible, or in the interior tissues of healthy plants and animals.

Bacterial multiplication takes place under most diverse conditions and bacterial multiplication involves the formation of cell-substance or protoplasm which, in turn, involves the synthesis of all the complicated concomitants of free-living existence such as proteins, amino-acids, carbohydrates, lipoids, nucleic acids, growth factors, prosthetic groups of enzymes, etc. Many of these substances can be synthesised in the laboratory only with extreme difficulty, if at all. Not only is there the synthesis of complex molecules to be accomplished but in many cases these syntheses are further complicated by considerations of positional isomerism which give rise to the formation of several substances of the same empirical formula, only one of which is biologically effective. To take a simple example, we have the amino-acid $R.CHNH_2.COOH$ which, as it contains an asymmetric carbon atom, exists in two isomeric forms, one the structural mirror-image of the other.

An examination of the alanine, $CH_3.CHNH_2.COOH$, of bacterial protoplasm will show that it consists almost entirely of the *laevo-* form, whereas the alanine synthesised in the laboratory will consist of equal parts of *laevo-* and *dextro-* forms. If the organism is given synthetic alanine as a source of nutrient, it will utilise the *laevo-* form, while leaving the *dextro-*alanine almost untouched, although it has been shown recently that very small amounts of D-alanine are taken up by the growing cells. If we hydrolyse the proteins of bacteria we find that they are composed almost entirely of the *laevo-* forms of the various amino-acids, although in some cases, as in the antibacterial peptides excreted by certain *Bacilli*, a proportion of the constituent amino-acids are found to be of the *dextro-* form. Some twenty odd amino-acids have been isolated and proteins consist of these amino-acids condensed in various permutations and combinations into peptide chains. Proteins differ in the order and sequence of the amino-acids in the chain, but so far the synthesis of any single protein has eluded the chemist in his laboratory.

Further complications of positional isomerism are met in structures of the nature of coenzyme I, adenine-nicotinamide-dinucleotide, which plays a part as a carrier of hydrogen in cellular respiratory processes. Analysis of hydrolytic and enzymatic degradation products of this substance leads to a structural formula:



but synthesis *in vitro* is made difficult by the facts that the whole molecule is essential for biological activity and that any alteration in the nature or position of the linkages around

the ribose molecules renders the substance inactive. The power to synthesise proteins, nucleotides, etc., is a property of many living tissues and in some bacteria we have organisms which are able to synthesise all these substances from very simple raw materials and at considerable speed. For example, the group of bacteria known as the chemosynthetic autotrophes synthesise bacterial protoplasm from purely inorganic sources, utilising CO_2, NH_3, and inorganic salts as the raw material from which is produced all that chemical complex forming the multiplying cell.

The synthetic abilities of bacteria form an absorbing problem for the chemist, although these abilities are not necessarily exceptional amongst living cells—we do not yet know sufficient about this aspect of metabolism to say how exceptional or unexceptional the synthetic powers of bacteria may be—but when we come to consider the destructive (catabolic) activities of bacteria we are faced with a bewildering diversity of chemical potential. It is common experience that when an organism dies and falls on to the surface of the earth, it will disappear in the course of time. Carcases and corpses are buried, dead plants and plant trimmings are composted, excreta are spread on open fields, and, in due course, they are altered into some form not recognisably related to the original. This is mainly due to the scavenging action of soil micro-organisms which by their destructive abilities break down the dead material and convert it into bacterial protoplasm (fungal protoplasm, etc.) and various soluble products. Think for a moment what this involves: chemically inert proteins such as keratin; polysaccharide complexes such as chitin and cellulose; fats, hydrocarbons, lipoids, sterols, etc., are broken down into simpler substances which are assimilated, putrefied, or fermented, with the resultant production of bacterial protoplasm, salts, ammonia, carbon dioxide, gaseous N_2 and H_2, etc. Bacteria which can oxidise sulphur to sulphuric acid exist in sulphuretted waters, others exist in soil deriving energy for existence from the oxidation of hydrogen to water, while

others are found in the soil around oil-wells which oxidise paraffin hydrocarbons to carbon dioxide and water. It is probably not unscientific to suggest that somewhere or other some organism exists which can, under suitable conditions, oxidise any substance which is theoretically capable of being oxidised.

Here then we have a small sample of the fascinating field of chemical activity presented by bacteria. There are many questions which immediately occur to the chemist. How do these micro-organisms carry out these reactions which cannot be achieved in the laboratory? Is it possible to utilise their activities to carry out such and such a reaction? Can their metabolic activities be exploited on a commercial scale? Why are some bacteria pathogenic to man? Bacterial metabolism has been studied ever since the initial investigations of Pasteur, and as new techniques are devised our knowledge is continually increasing and accumulating, but it is still true to say that we understand only a very small part of the activities of bacteria and there is immense scope for research in this field. In this book an attempt will be made to answer some of the queries that arise in the mind of the chemist, and in many cases the answers will be such that they will merely indicate our need for further research.

Chemical reactions carried out by living material take place in simple steps and these steps can often be demonstrated within the cell either by suitable treatment of the cell, or by the addition of chemicals which will combine with intermediate products or with enzymes involved in the formation of these products, and so break up complete reactions into their individual steps. The number of basic reactions is few and include the following:

1. *Reduction:* the addition of hydrogen or, alternatively, the removal of oxygen from the molecule attacked.
2. *Oxidation:* the removal of hydrogen or, alternatively, the addition of oxygen.
3. *Dehydration:* the removal of H_2O from the molecule.

4. *Hydrolysis:* the addition of H_2O to the molecule, a step which is usually followed by a splitting of the molecule at the link hydrolysed.
5. *Deamination:* the removal of $-NH_2$ from the molecule.
6. *Decarboxylation:* the removal of CO_2 from -COOH.
7. *Phosphorylation:* the esterification of the molecule with phosphoric acid—usually accomplished by the transfer of the phosphate radicle from some substance other than phosphoric acid itself.
8. *Dephosphorylation:* the removal by hydrolysis of phosphoric acid from phosphorylated compounds.

These eight possibilities may all be utilised in the attack on a given molecule by different bacteria. It is the fact that different bacteria can and do utilise different methods of attack on the same substrate molecule that gives rise to the varied products of bacterial activity and to the apparently involved and complicated metabolism of the order as a whole.

The metabolism of the cells of highly organised tissues living in a constant environment, such as those of the mammalian body, seems to be simple compared with that of bacteria which live in varied environments. The blood of the rat does not differ greatly from the blood of man and the metabolism of a rat muscle-cell, rat liver-cell, or rat kidney-cell does not differ greatly from the metabolism of human muscle-, liver-, or kidney-cell, or from the metabolism of similar cells in another rat. But the metabolism of a cell in a culture of *Escherichia coli* may differ greatly from that of a cell of the related *Aerobacter aerogenes* or even from that of a cell of another culture of *Escherichia coli* grown under different conditions. The metabolism of the bacterial cell is dependent not only on the intrinsic or potential composition of the organism but also on the environmental conditions holding during its division from the mother-cell. To take an example, consider the molecule of pyruvic acid, $CH_3.CO.COOH$. The muscle-cells of

man, rat, frog, etc., will reduce this to give lactic acid, $CH_3.CHOH.COOH$. A culture of *Aerobacter aerogenes* grown at *p*H 8 will attack it by hydrolysis to give acetic and formic acids:

$$CH_3.CO.COOH + H_2O = CH_3.COOH + H.COOH.$$

The same organism grown in the same medium but adjusted to *p*H 6 will utilise a third method of attack by decarboxylation:

$$2CH_3.CO.COOH = CH_3.CO.CHOH.CH_3 + 2CO_2,$$

while another organism, *Propionibacterium*, will reduce pyruvic acid to propionic acid $CH_3.CH_2.COOH$. The fact that a given culture of a bacterium will attack a certain substance in a certain way means nothing more than that a culture of that identical organism grown and tested under identical conditions will attack that substance in that way; vary the growth conditions, the experimental test conditions, the strain, species, genus, or family of organism and we cannot say, without further experiment, anything about the reaction that will occur. At first sight it would seem as though we have here a biological problem which is uncontrollable from a chemical point of view by reason of its possible variations. But the situation is not as hopeless as it may first seem; the nature of the attack on a given substance by a bacterium depends upon

1. the bacterium,
2. the conditions under which it grows,
3. the conditions under which it is tested.

By taking typical organisms and studying their chemical reactions under various growth and experimental conditions, we have already acquired a considerable amount of knowledge concerning the factors governing the variations under (2) and (3). Once we have covered the ground with one organism, we can repeat with others closely and distantly related, and, fortunately, we often find that there are certain fundamental principles underlying the variation of activity with environment.

It will be seen as we go along that it is often possible to predict how a given organism may react to a given chemical environment or how to arrange the chemical environment in such a way that bacteria might be expected to carry out a desired chemical task—although only experiment will tell whether they will actually do so.

Before embarking on this problem we must first have some understanding of the reasons why bacteria attack their environment at all. When an organism is inoculated into a suitably nutrient medium, it begins to grow, synthesising new bacterial protoplasm with consequent increase in size until eventually division takes place with the formation of two cells from one. The rate at which subsequent divisions occur depends to a large extent upon the nature of the medium but an organism such as *Escherichia coli* living in a rich medium such as a tryptic digest of casein can divide once every ten or fifteen minutes. At this rate one organism can give rise to over one million organisms in five hours. Consequently one organism can synthesise over one million times its own weight of bacterial protoplasm in five hours. This high rate of synthesis must take place at the expense of the environment which has to supply all the raw materials including major requirements of carbon and nitrogen; minor requirements of phosphorus, sulphur, and iron, and traces of many other elements. Since these elements may be present in the medium in a form not primarily utilisable by the organism, it must attack the complex substances present in the medium so as to render the raw materials available in an assimilable and utilisable form. Secondly, the synthesis of this chemical complex of the bacterial cell involves the expenditure of energy and this the organism obtains by the degradation of energy-rich substances in the environment. Thirdly, if the physico-chemical properties of the environment vary to any significant extent during the synthesis, then the organism reacts by speeding up those reactions tending to stabilise the internal environment. For example, the decomposition of carbohydrate for energy purposes usually results in

the formation of acid end-products and a consequent fall in the *p*H of the medium, which may be of such dimensions as to sterilise the activities of the growing organism; under such conditions some organisms are capable of catalysing neutralisation reactions which either have alkaline end-products or result in the alteration of acid products to neutral ones, so that some degree of stabilisation of the internal environment is accomplished. The organisms therefore attack their environment to obtain material for growth, energy for synthesis, and stabilisation against unfavourable conditions.

CHAPTER II

THE NATURE AND IDENTIFICATION OF BACTERIA

Bacteria

Bacteria are simple unicellular organisms which multiply, often very rapidly, by binary fission. The majority possess no chlorophyll, though bacterial chlorophyll does occur in the photosynthetic organisms (see p. 86). No nucleus is visible in the bacterial cell if this is examined by the usual methods, although the application of particular staining techniques will reveal the presence of what are usually called " nuclear structures " in the cytoplasm.

Size

Large bacteria such as *Clostridium welchii* may have a length as great as 3-5μ (1μ = 0·001 mm.) and, at the other end of the scale, we have various Micrococci with a diameter of 300-500 mμ (1mμ = 0·001μ). The larger organisms thus approximate in length to half the diameter of the red blood-corpuscle or of the yeast-cell. At the other end of the scale it is difficult to draw a line between the smaller bacteria and the larger viruses, especially if we take into account the *Rickettsia* which, in size and properties, fall between the true bacteria, which can exist outside the cells of a host, and the viruses which can multiply only within the cells of a host. The frontispiece shows the gradation in size from *Cl. welchii*, streptococci, rickettsia, and viruses large and small, to protein molecules. Most bacteria can be cultivated in laboratory media of varying complexity, but in general it is true to say that the smaller the organism, the poorer its synthetic powers and, consequently, the more parasitic it becomes, until in the ultimate stages shown by the viruses a sufficiently nutrient medium is supplied only in the interior of the living cells of a host. A particle having the size of the Foot-and-Mouth-disease virus is sufficiently large to accommodate about 50-100 protein molecules only.

IDENTIFICATION

General

An organism is identified by a consideration of many properties including its shape, staining reactions, biochemical reactions, pathogenicity, etc. Many of the properties of an organism tend to change with conditions of cultivation, such as the nature of the growth medium, the age of the culture, the temperature, the degree of aerobiosis, etc., and consequently classification must be based as far as possible upon stable properties tested under standard conditions. There is no point in this book in attempting a detailed account of the theory or practice of systematic classification, but the non-bacteriologist requires some guidance concerning the identification of particular organisms, so the following represents a brief account of the properties which are investigated for purposes of classification.

Morphology

Some bacteria are spherical, some rod-shaped, comma-shaped, or twisted like a spiral, and all varieties of intermediate shapes occur. The shape, where it is constant, is easily observed through the microscope and formed the basis of many of the earlier systems of classification. Thus spherical organisms were called "Cocci," rod-shaped organisms "Bacilli," and spiral-shaped "Spirilla." This simple morphological grouping has nowadays been complicated by subdivision of the groups on a basis of other characteristics.

Spore formation

Some bacteria possess the capacity to produce spores which are a resting or non-vegetative form considerably more resistant to heat, desiccation, or unfavourable chemical environments than the vegetative forms. It was thought at one time that these organisms form spores when their environment becomes unsuitable for continued vegetative existence but this is not necessarily the case, as it is known that some

organisms will form spores only if their environment is nutritionally rich, and spore formation then appears to be part of the normal life-cycle of the organism. Whatever may be the cause of their formation, spores constitute a form in which the organism can survive for long periods under adverse conditions. When the environment again becomes suitable for vegetative existence, the spores germinate to form normal cells capable of multiplication as usual.

Staining reactions

A useful test that can be applied to bacteria as an aid in diagnosis is their reaction to the staining technique invented by Christian Gram. The dried organisms (in the form of a smear on a microscope slide) are stained with a dye of the pararosaniline series (see p. 203) such as crystal violet and then treated with iodine solution. The preparation is then washed with alcohol until no more violet dye washes off the slide and finally counterstained with a dye of contrasting colour—usually a red dye such as carbolfuchsin. Under this treatment some organisms retain the violet dye and are said to be " Gram-positive," whilst the violet dye is washed out of others by the alcohol, these are stained red by the counterstain and are said to be " Gram-negative." It has been shown recently that the staining complex in Gram-positive organisms is a nucleoprotein which can be extracted from the cells which then stain Gram-negative. For some reason not yet understood, those organisms which are Gram-positive differ in general—there are individual exceptions—from the Gram-negative organisms in being more exacting nutritionally (see Chap. V), having more restricted chemical activities, and in being more sensitive to the action of chemotherapeutic agents such as penicillin, the sulphonamides, the triphenylmethane dyes, and the acridine dyes.

Cultural characteristics

The nature and composition of the medium in which the organism will or will not grow may aid its identification (see

Chap. V). When a satisfactory growth medium has been obtained, further assistance in identification can be obtained from the investigation of colony form. The medium is mixed with agar-agar, sterilised, and poured while hot into Petri dishes; on cooling, the medium solidifies as a sheet of nutrient jelly. The organisms, in high dilution, are streaked on to the surface of the solid medium, the dish covered, and then incubated. Each organism on the medium proceeds to multiply and to form a small pile or " colony " which, after 24-48 hours, is visible to the naked eye as a tiny stud or convexity on the surface of the medium. Each single colony may represent a pure culture in that it has arisen from a single organism and, if the cells are far enough apart at inoculation, then discrete and distinct colonies will appear on the plate. Colonies of different organisms have different appearances: *Esch. coli* gives smooth, round, translucent colonies; *Streptococcus faecalis* on media containing glucose forms small, round, white colonies; *Staphylococcus aureus* forms round, raised colonies which turn golden-yellow or orange after 48 hours incubation; *Serratia marcescens* gives blood-red colonies; while *Proteus vulgaris*, which is highly motile, forms big, flat, spreading colonies that look like mountain ranges on a contour map.

Oxygen requirement

Bacteria fall into four main groups according to the oxygen tension they can tolerate for growth:

1. Strict aerobes: organisms which can multiply only in the presence of oxygen.
2. Facultative anaerobes: organisms which can live equally well in the presence or complete absence of oxygen.
3. Microaerophilic organisms: organisms which can live in the absence of oxygen or in the presence of very low oxygen tensions, high tensions being inhibitory.
4. Strict anaerobes: organisms which can multiply only in the complete absence of oxygen.

The nature of the metabolism of an organism is closely connected with the aerobic or anaerobic nature of its growth conditions.

Biochemical characteristics

This book is mainly concerned with the variety of chemical changes that bacteria can produce in their environment. Where these changes can be detected easily they can often be used to separate individuals which appear to be alike in morphological and other characteristics. This will be dealt with in greater detail below.

Serological characteristics

When a foreign body, particularly if it is of protein nature, is introduced into the blood-stream of an animal it may there act as an antigen and stimulate the animal to produce antibody. The serum of the animal will then contain the antibody which will react specifically with the antigen. Bacterial cells are antigenic and if we inject bacterial cells into an animal, the serum of that animal will eventually contain antibody which will react with the cells *in vitro* so as to produce a visible result such as agglutination. Since the antibody formed in response to the injection of an antigen is specific for that antigen, the reaction can be used as a delicate test for that antigen. The surface of the bacterial cell may contain several different antigens and the composition of the surface varies from one organism to another. Consequently the serum prepared as a result of the injection of an organism A will react with cells of A itself or of organisms possessing the same antigen in their surface. By preparing the antibody to A we can therefore determine what other cells belong to the same antigenic group or, alternatively, we can divide a collection of organisms into groups according to their antigenic reactions. For example, the species *Streptococcus haemolyticus* has been divided by Lancefield into a number of groups, known as the Lancefield Groups A, B, C, D, etc., by serological methods. By a modification

of the method it has been possible to divide the groups still further into serological types so that, for example, Lancefield Group A *streptococci* have been divided into some thirty-two serological types.

Animal inoculation

The medical bacteriologist dealing with pathogenic organisms has a further possibility of characterising an organism by the lesions it produces after inoculation into a suitable animal host. Thus the tubercle organism can be identified by the lesions it produces after injection into a guinea-pig.

CLASSIFICATION

By combinations of the tests outlined above it is possible to separate organisms into groups and sub-groups. There will always be individuals which will not fit cleanly into any set grouping, but the majority can be assigned to various pigeon-holes in a systematic classification. In some cases the outstanding characteristics will be morphological, cultural, or pathogenic, while in others differentiation will be based upon finer investigation of a multiplicity of biochemical reactions. Many systems of classification have been used in the past and there is, unfortunately, no definite agreement upon any one system at the present time. The nomenclature used in this book is that adopted by the Society of American Bacteriologists and detailed in Bergey's *Manual of Determinative Bacteriology*. In Table I the names of the main Families, Tribes, and Genera used in this book are outlined, but for details concerning the rationale of the classification and for the differentiation of the groups, reference must be made to the *Manual* and other standard textbooks of bacteriology.

ORDERS AND FAMILIES

The whole group of micro-organisms which come within our description of "bacteria" is strictly termed *Schizomycetes* (fission-fungi) and is divided into several orders. Most

TABLE I

OUTLINE OF SYSTEMATIC CLASSIFICATION OF THE ORDER EUBACTERIALES

Family	Tribe	Genus
I. Nitrobacteriaceae	Nitrobacterieae	Nitrosomonas Nitrobacter
	Thiobacilleae	Thiobacillus
II. Pseudomonadaceae	Pseudomonodeae	Pseudomonas Acetobacter
	Spirilleae	Vibrio Desulphovibrio Spirillum
III. Azotobacteriaceae		Azotobacter
IV. Rhizobiaceae		Rhizobium
V. Micrococcaceae		Micrococcus (Staphylococcus) Sarcina
VI. Neisseriaceae		Neisseria Veillonella
VII. Lactobacteriaceae	Streptococceae	Diplococcus Streptococcus Leuconostoc
	Lactobacilleae	Lactobacillus Propionibacterium
VIII. Corynebacteriaceae		Corynebacterium
X. Enterobacteriaceae	Escherichieae	Escherichia Aerobacter
	Serrateae	Serratia
	Proteae	Proteus
	Salmonelleae	Salmonella Shigella Eberthella
XII. Bacteriaceae		Bacterium Methanobacterium
XIII. Bacillaceae		Bacillus Clostridium

biochemical studies have been carried out with members of the order *Eubacteriales* in which the organisms exist as separate individuals and do not show any form of mycelium or filaments. The order is subdivided into families, partly on a morphological basis and partly on a chemical basis. Eleven of the families differentiated by Bergey are included in Table I. The *Nitrobacteriaceae* comprise organisms which can carry out an oxidation of inorganic material as source of energy. The *Azotobacteriaceae* and the *Rhizobiaceae* are both capable of utilising atmospheric nitrogen as nitrogen source, the latter carrying out the fixation process only when living in symbiosis with a host-plant (see Chap. X). The *Micrococcaceae* are usually Gram-positive spherical organisms, while the Gram-negative coccal organisms are placed in the *Neisseriaceae*; the spherical organisms which divide to form chains, the *Streptococci*, are not included in these groups but are classed in the *Lactobacteriaceae* since they carry out a simple lactic acid fermentation of glucose, and generally resemble the *Lactobacilli* in their nutritional and chemical characteristics. The rod-shaped organisms are divided into several families: the *Bacillaceae* being those organisms which can form spores under suitable conditions, the non-sporing rod-shaped organisms are subdivided and most of the organisms of this group with which we shall be dealing in this book are classed in the *Enterobacteriaceae* which comprises many of those bacteria normally found in the intestinal flora.

TRIBES AND GENERA

In many cases families include organisms which fall clearly into sub-groups. Thus the *Lactobacteriaceae* are subdivided into spherical organisms, *Streptococceae*, and rod-shaped organisms, *Lactobacilleae*. Again, within the family *Nitrobacteriaceae*, we have organisms using inorganic nitrogenous substances as oxidation substrate, the *Nitrobacterieae*, and other organisms, obtaining energy from the oxidation of sulphur compounds, which are consequently placed in a

separate "tribe" called the *Thiobacilleae*. The *Enterobacteriaceae* are divided into a number of tribes, some of which are shown in Table I; the divisions in this case are based on less well-defined characteristics and are still the subject of debate amongst taxonomists.

The next subdivision of the tribe is into genera. Again the basis of subdivision may be morphological as in the separation of *Streptococci* which are spherical organisms dividing about one axis to form long chains, from *Diplococci* in which the organisms occur in pairs rather than in chains. The separation of genera within the *Micrococcaceae* is similar: when division takes place evenly about three axes to give cubical packets the organisms are called *Sarcina*; when division occurs about two axes to give plates the organisms are called *Micrococci*; a third genus, *Staphylococcus*, used to comprise organisms dividing unevenly to give "bunches of grapes," but the latest edition of Bergey includes the *Staphylococci* within the *Micrococci* as "variants." In the *Bacillaceae* two genera are differentiated by oxygen tolerance, thus the *Bacilli* are strict aerobes and the *Clostridia*, strict anaerobes. In many cases the differentiation of genera within a tribe is based upon biochemical characteristics; thus the *Lactobacilli* ferment glucose to produce lactic acid only, while the *Propionibacteria* have a more varied array of fermentation products including propionic acid. The differentiation between *Escherichia* and *Aerobacter* also rests largely on differences in fermentation (see Chap. VII).

SPECIES

An organism can be allocated to a family and genus along the lines already indicated, but the differentiation of species within many genera is mainly a matter of biochemistry, that is, the species are separated by their reactions in a number of simple biochemical tests. If we examine the fermentation of a number of sugars such as sucrose, glucose, maltose, fructose, etc., by different organisms, we shall find, without going into any detailed examination of the products, that some organisms can ferment some sugars but not others. Further, if we add

indicator to our sugar media and immerse a small inverted tube full of media in the culture-tube, then we shall find that the fermentation of a given sugar by one organism gives rise to acid as shown by a change in the colour of the indicator, while fermentation of the same sugar by another organism gives rise to acid and also gas as shown by the accumulation of bubbles inside the inverted tube. If we extend our range of fermentation substrates to include such as xylose, mannitol, dulcitol, glycerol, salicin, etc., we shall find that we have already a method for the separation of certain individuals from others by the range and nature of their fermentations. With these tests we can combine others which involve simple manipulation and observation, such as those for the formation of indole from protein or amino-acid media (see p. 172) or for the formation of acetylmethylcarbinol during the fermentation of glucose (see p. 136). By using a series of such tests we find that it is possible to differentiate many common organisms, as can be seen from the selection of organisms and their reactions in Table II (see p. 22).

The following are some of the common species mentioned in this book:

Family I

NITROSOMONAS species all utilise ammonia as source of nitrogen and energy. These organisms are very difficult to isolate in pure culture and no methods of separating species have yet been described.

NITROBACTER species utilise nitrite as nitrogen source and cannot utilise ammonia.

THIOBACILLUS THIO-OXIDANS is found in sulphur-containing soils and waters, and obtains energy by the oxidation of sulphur to sulphuric acid, to which it is very resistant.

Family II

PSEUDOMONAS AERUGINOSA (PYOCYANEA) is a common soil organism which produces a distinctive blue-green pigment, pyocyanine, which is excreted into the medium. It is non-pathogenic but proves to be extremely difficult to eradicate from wounds.

ACETOBACTER species are found in the vinegar industry and as contaminants in brewing vats. *Acetobacter xylinum* produces a form of capsule made of cellulose.

DESULPHOVIBRIO DESULPHURICANS can be isolated from mud and sulphur-containing waters. It is a comma-shaped organism which, in some media, grows to give spiral-shaped organisms of considerable length and marked motility. It reduces sulphate to hydrogen sulphide.

Family V

MICROCOCCUS PYOGENES VAR. AUREUS (STAPHYLOCOCCUS AUREUS) is the common organism producing pus in wounds, boils, etc. It is a spherical organism producing orange colonies on solid media. It is one of the organisms which cause septicaemia and ostiomyelitis.

Family VI

NEISSERIA INTRACELLULARIS (Meningococcus) is the causal organism of meningitis.

NEISSERIA GONORRHOEAE (Gonococcus) is the causal agent in gonorrhoea.

Family VII

STREPTOCOCCUS HAEMOLYTICUS, the causal organism of scarlet fever, streptococcal septicaemia, puerperal fever, and streptococcal throat. The organism causes lysis of red blood-cells by secretion of a haemolysin and the species has been divided into serological groups and types; the human pathogens belong mainly to group A.

STREPTOCOCCUS FAECALIS, one of the common intestinal inhabitants. It is non-pathogenic although some variants are haemolytic and belong to the haemolytic group D.

STREPTOCOCCUS LACTIS, the common non-pathogenic streptococcus of milk. This organism can be clearly differentiated from *S. faecalis* by biochemical and serological tests. Both *S. faecalis* and *S. lactis* are used for nutritional assay procedures (see p. 110).

LACTOBACILLUS CASEI, one of several species used for nutritional studies and normally found in milk.

Family VIII

CORYNEBACTERIUM DIPHTHERIAE, the causal organism of diphtheria.

Family X

ESCHERICHIA COLI, numerically the most common intestinal bacterium. This organism is easily grown in large quantities, is non-pathogenic, has very wide chemical activities, and has consequently been subjected to more intense biochemical investigation than any other bacterium.

AEROBACTER AEROGENES, an organism whose chemical activities are similar to those of *Esch. coli* but which is more commonly found in association with soil and plant materials than in intestinal contents.

PROTEUS VULGARIS, a highly motile soil organism usually found in association with putrefying material. It often proves a nuisance when it becomes established in wounds since, although it is non-pathogenic, it is insensitive to almost all the present antibacterial agents used in chemotherapy.

SALMONELLA species are food-poisoning organisms which produce toxins when growing in protein-containing media such as meat products, egg powders, etc.

SHIGELLA species cause dysentery if they become established in the intestinal flora.

EBERTHELLA TYPHOSA is the causal organism of typhoid.

Family XIII

BACILLUS SUBTILIS is a common air and soil inhabitant. Organisms of this and related species are now proving fruitful sources of new antibiotics.

BACILLUS ANTHRACIS is the causal organism of anthrax.

CLOSTRIDIUM TETANI is the causal organism of tetanus.

CLOSTRIDIUM BOTULINUM is the food-poisoning organism causing botulism. Its exotoxin is the most toxic substance known.

CLOSTRIDIUM WELCHII is an intestinal organism which, if it becomes established in a wound, produces a number of toxins and gives rise to the condition known as gas gangrene.

CLOSTRIDIUM ACETOBUTYLICUM is non-pathogenic and is used commercially for the production of acetone and butyl alcohol.

STRAINS

Using the chemical, morphological, and cultural tests described above it is possible to divide organisms into genera and species, but we cannot identify any particular strain of organism with certainty. For example, any organism having the characteristics in Table II (plus such others as are commonly used for finer differentiation) of, say, *Esch. coli* will be called "*Esch. coli.*" But organisms conforming to the *Esch. coli* test form the bulk of the flora of faecal matter and we can isolate millions of "*Esch. coli*" from a particle of faeces. Although these organisms all give the characteristic tests it does not follow that they are all identical or have sprung from one common stock. When we come to examine enzyme systems other than those involved in the systematic tests, or if we examine the *rates* at which the various sugars, etc., are attacked, we shall find wide differences between the various organisms that have been isolated and called "*Esch. coli*" and we say that these different organisms are different "strains" or "variants" of *Esch. coli.* Strains are often

TABLE II

Identification Tests for some Common Bacteria

Organism	Shape	Gram-stain	Growth		Spores	Patho-genic	Indole.	Fermentations							
			aer.	anaer.				VP.	Gl.	Lt.	Su.	Dl.	Mn.	Mt.	Sl.
S. haemolyticus ...	Sphere chains	+ ve	+	+	–	+	–	–	A	A	A	–	–	A	A
Staph. aureus ...	Sphere clusters	+ ve	+	+	–	+	–	–	A	A	A		A	A	–
Esch. coli	Rod	– ve	+	+	–	–	+	–	AG	AG	–	AG	AG	AG	
Aerobact. aerogenes ...	Rod	– ve	+	+	–	–	–	+	AG	AG	AG	–	AG	AG	
Eber. typhosa ...	Rod	– ve	+	+	–	+	–	–	A	–	–	A	A	A	
Pr. vulgaris	Rod	– ve	+	+	–	–	V	–	AG	–	V	–	–	V	
B. subtilis	Rod	+ ve	+	–	+	–	–	+	A	–	A	–	–	A	A
Cl. tetanum	Rod	+ ve	–	+	+	+	–	–	–	–	–	–	–	–	–
Cl. welchii	Rod	+ ve	–	+	+	+	–	–	AG	AG	AG	–	–	AG	–

Abbreviations:

VP — Voges-Proskauer test for acetylmethylcarbinol (see p. 136)
V — variable result
A — acid produced
AG — acid and gas produced

Gl. — glucose
Lt. — lactose
Su. — sucrose
Dl. — dulcitol

Mn. — mannitol
Mt. — maltose
Sl. — salicin

identified by the name of the person who first isolated them, thus: *Cl. septicum* Pasteur. Whenever we isolate a new *Esch. coli* we must assume that it is a new strain different from any other, as two cultures cannot be said to be of the same strain unless they have identical chemical properties, qualitatively and quantitatively, under all possible conditions of growth. In practice we never assume that two organisms are of the same strain unless we know that they have both been cultivated from the same mother-culture (or ideally from the same mother-cell), and even then it is not uncommon for an organism to give rise to two or more strains by mutation in the course of serial subcultivation. The chemist working with bacteria must be careful to specify not only the species but also the strain of any organism used for a given purpose, and it does not follow that a published experiment can be repeated unless the identical strain used in the original work is used for the repetition. The relation between serological types and biochemical strains has not yet been sufficiently clarified for any general statement to be made; it is probable that a group of organisms belonging to one serological type would contain strains separable on biochemical grounds.

Abbreviations

The standard abbreviations for generic names have been adopted in this book: *Bact.* for *Bacterium*, *B. for Bacillus*, *Cl.* for *Clostridium*, *Esch.* for *Escherichia*, *Pr.* for *Proteus*, *Ps.* for *Pseudomonas*, *Staph.* for *Staphylococcus*, *S.* for *Streptococcus*. Where genera are mentioned or species not included in Table I, these have been given their full titles.

FOR FURTHER READING

Manual of Determinative Bacteriology, Bergey (Baillière, Tindall and Co.).

Handbook of Practical Bacteriology, Mackie, T. J., and McCartney, J. E. (Livingstone).

The Bacterial Cell, Dubos, R. (Harvard University Press).

Fundamentals of Bacteriology, Frobisher, M. (W. B. Saunders).

CHAPTER III

BACTERIAL ENZYMES

Enzyme action

The chemical activities of bacteria and other living tissues are due to the catalytic action of enzymes. Enzymes are organic substances which are produced by living cells and which act as catalysts of specific reactions. They have properties similar to those of catalysts used in chemical processes in that they speed up the velocity of a reaction without altering the nature or proportions of the products and without adding any energy to the reacting system. Other things being equal, the velocity of the reaction is proportional to the concentration of the catalyst or enzyme. Enzymatic activity is dependent upon certain physical conditions being suitable; thus an enzyme is active over a small range of *p*H only, the value at which it displays maximal activity being known as the "optimal *p*H." For the majority of enzymes this optimal *p*H value lies between *p*H 5 and 8, but in individual cases it may be within wider limits of 2-10. A catalyst can speed up a reaction which is already proceeding slowly, but it cannot by itself initiate a reaction, and it is as yet uncertain whether this is or is not the case with enzymes. Many reactions which take place in the presence of living tissues will not noticeably take place in their absence, but, as has already been pointed out, many chemical reactions occur at too slow a rate to be perceived by man through his senses. It is not illogical therefore to regard enzymes as catalysing reactions which are normally occurring at an insensible rate, but many authorities are of the opinion that enzymes, by straining the structure of the molecule upon which they act and so rendering it more susceptible to change, can initiate new reactions. Enzymes also differ from most

chemical catalysts in that they are thermolabile or destroyed by heat. A number of enzymes have now been isolated in a pure and even crystalline state and are found to be proteins the structure of which is altered (or denatured) by heat.

The substance whose chemical change is catalysed by an enzyme is said to be the "substrate" of that enzyme and the majority of enzymes display a strict specificity towards their substrate. This specificity may be such that the enzyme can catalyse the alteration of one substance only; thus succinic dehydrogenase is an enzyme which will catalyse the removal of hydrogen from succinic acid to form fumaric acid:

$$\begin{array}{l} CH_2.COOH \\ | \\ CH_2.COOH \end{array} \quad \begin{array}{c} \\ -2H \longrightarrow \\ \end{array} \quad \begin{array}{l} CH.COOH \\ \| \\ CH.COOH \end{array}$$

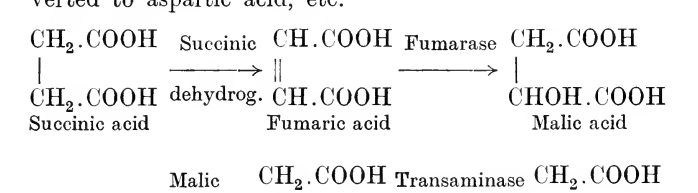
(Succinic dehydrog.)

but the enzyme is specific towards succinic acid and will dehydrogenate no other substance. Where the substrate exists in two or more isomeric forms the enzyme is usually specific for one isomer only; thus L-lysine decarboxylase will decarboxylate L-lysine to cadaverine but is unable to attack D-lysine or any other amino-acid:

$$\underset{\text{L-lysine}}{H_2N.CH_2.(CH_2)_3.CHNH_2.COOH} - CO_2 \longrightarrow \underset{\text{Cadaverine}}{H_2N.CH_2.(CH_2)_3.CH_2.NH_2}$$

Such optical specificity has been used in the past for the resolution of racemic mixtures since the enzyme will attack one isomer and leave the other intact. The specificity may, on the other hand, be less restricted so that an enzyme may catalyse the alteration of any substance containing a certain chemical grouping; thus some proteases may hydrolyse any substance having the linkage —CO—NH— in its structure and will hydrolyse proteins and peptides down to amino-acids. Other proteases, however, will attack the peptide link only if the residues R and S on either side of the link R—CO—NH—S have specific structures.

This specificity of enzyme action is important in that it means that an organism such as a bacterial cell must possess enzymes for each process it carries out at a rate greater than that which will occur in the absence of living cells. Consequently an organism which carries out numerous different chemical reactions must contain numerous enzymes in its constitution. If an organism possesses succinic dehydrogenase it will dehydrogenate succinic acid to fumaric acid (or, alternatively, since the reaction is a reversible one, will hydrogenate fumaric to succinic acid in the presence of a hydrogen-donator). If it possesses an enzyme called "fumarase" it will hydrate fumaric acid to L-malic acid; if it possesses L-malic dehydrogenase, malic acid will be dehydrogenated to oxalacetic acid; if it possesses transaminase then oxalacetic acid can be converted to aspartic acid, etc.



So we get a chain of reactions in which each step is catalysed by a specific enzyme. If any one of these enzymes is missing then the chain stops at the substance forming the substrate of the missing enzyme. The distribution of enzymes among bacteria is very varied and, taking this chain of reactions as example, bacteria exist in which four, three, two, one, or none of these enzymes are present and consequently the end product of succinic acid metabolism in various organisms may be succinic acid, fumaric acid, L-malic acid, oxalacetic acid, or L-aspartic acid. Here then lies the key to the variety of chemical reaction displayed by bacteria; every substance that is metabolised passes through a chain of small alterations, each one involving a simple chemical change and each catalysed

by a specific enzyme the distribution of which is by no means universal.

The nature of enzyme action means that if a reaction is a reversible one, then the enzyme-catalysed reaction is also reversible and the back reaction will be catalysed to the same extent as the forward reaction so that the final equilibrium mixture is the same whether the whole reaction is catalysed or not. This means that the degree to which a given metabolite in a chain of reactions will accumulate will depend upon the velocity of the forward and backward reactions of the various steps in the chain, and all the substances in one chain may be side-tracked into another chain by an alteration in the conditions governing the various equilibria. Where a given molecule may be dehydrogenated in organism A, it may be hydrolysed in organism B with the production of a different chain of reactions. Taking the above series of reactions as example again, *Esch. coli* possesses an enzyme " aspartase " which brings about a reaction between fumaric acid and ammonia to produce aspartic acid without the other intermediate steps:

$$\begin{matrix} CH.COOH \\ \| \\ CH.COOH \end{matrix} + NH_3 \xrightleftharpoons{\text{Aspartase}} \begin{matrix} CH_2.COOH \\ | \\ CHNH_2.COOH \end{matrix}$$

Other organisms possess an oxalacetic decarboxylase which decarboxylates oxalacetic acid to pyruvic acid and that opens up an enormous number of possible reaction chains (see Chap. VII).

$$\begin{matrix} CH_2.COOH \\ | \\ CO.COOH \end{matrix} - CO_2 \xrightarrow[\text{decarboxylase}]{\text{Oxalacetic}} \begin{matrix} CH_3 \\ | \\ CO.COOH \end{matrix}$$

The final product of succinic acid metabolism in an organism will depend therefore upon, first, the enzymes present in the organism and, second, upon the various side reactions occurring at the particular moment studied. The chemical variety of bacterial action is therefore based upon the permutations and

combinations of the enzymes of the organism and their interplay with the external environment.

THE STUDY OF ENZYMES IN BACTERIA

When organisms are growing in a medium, their chemical activities involve the building of cellular material and the breakdown of substances in the medium; thus we may have one sort of protein being hydrolysed outside the growing cell and another sort being synthesised within the cell. The number of enzymes concerned may be very great and their integrated activities too complex to disentangle. The first step in the simplification of the system is to eliminate synthetic reactions by preventing growth; this is performed by removing the cells from the growth medium, washing them free from traces of medium, and then suspending the washed cells in distilled water or a suitable salt solution. Investigations of the activity of bacterial enzymes are usually carried out in the first place with such " washed suspensions." Bacteria are seldom susceptible to osmotic rupture when suspended in water, and washing the cells in water often has no deleterious effect upon their chemical activities, but if the properties of the cell-wall are involved in these activities, then it is preferable to wash the cells in a salt solution of composition similar to that of the medium in which they were grown. Since many organisms retain their enzymic activities unimpaired in such " washed suspensions " they can be used for the investigation of metabolic changes, and the system further simplified by incubating the suspension with a single substrate in the presence of a known buffer solution. It does not follow that washed suspensions can be used to study all the enzyme systems of an organism, as it is sometimes found that some activities " decay " rapidly after, or during, the preparation of the suspension. It is seldom that suspensions can be kept in an active state for more than twenty-four hours, although some enzymes, *e.g.* formic dehydrogenase, will remain active for weeks even in autolysing suspensions.

The enzymatic activities studied in intact cell suspensions may be complicated by such factors as the rate of passage of the substrate through the cell-wall, the removal of reaction products by other enzyme systems, and differences between the physico-chemical conditions holding inside and outside the cell. Also with complex reactions which may involve many small changes, each catalysed by a specific enzyme, it is difficult to determine whether a given change is the result of the action of one or more enzymes. This can only be decided by preparing the enzymes in a cell-free state, studying their action *in vitro*, and separating them by methods of protein separation and purification. Where bacteria produce extracellular enzymes, these can be easily prepared in the cell-free state by filtering the cells from the medium and then removing the enzyme from the filtered medium by precipitation or by adsorption on to a suitable adsorbant such as alumina or calcium phosphate gel. The study of intracellular enzymes involves rupture of the cell-wall prior to purification procedures. The cell-walls of bacteria are very resistant and special methods have been devised to rupture them in such a way as to liberate the enzymes in an active state. The methods that can be used depend to a certain extent upon the relative resistance of the enzyme concerned and of the cell-wall to the treatment. The following are some of the more common methods used:

1. Thick washed suspensions of cells are treated with denaturing agents such as toluene, acetone, acetone-ether mixtures, or by simple drying. If the enzymes survive such treatment they can often be extracted from the denatured cell debris by incubation in buffer solutions.

2. The cell suspensions are incubated with proteolytic enzymes such as pepsin, trypsin, or papain and the debris extracted with buffer solutions.

3. Mechanical disintegration in some form of ball-mill. The simple ball-mill is usually ineffective but an effective crushing-mill consisting of steel cylinders rotating in a race

under pressure has been devised by Booth and Green.[1] A simpler mill has recently been produced in which a steel ball runs in a closely fitting channel in a steel bowl, the whole being immersed in solid CO_2; the combined effect of pressure and freezing accomplishing disintegration of the cells.

4. Disintegration by friction between fine hard particles. Werkman and his colleagues[2] first showed that thick pastes of bacterial cells can be disintegrated by grinding with finely powdered glass in a mortar. They later evolved a mechanical mortar to deal with large quantities of organism. Other workers have found that powders of carborundum or alumina are as effective as glass.

5. Disintegration by vibration. Exposure of cell suspensions to supersonic vibration of a certain range of frequency results in very effective breakdown of the cell structure, in fact, care has to be taken to prevent breakdown of the enzyme structures themselves. Vibration of lower frequency is often effective (*i.e.* sonic vibration of 50-60 cycles/sec.) but the efficiency is usually increased by addition of small glass beads or carborundum particles to the cell material.[3]

6. Specific treatment can be applied to certain organisms. Thus the enzymes of *Micrococcus lysodeikticus* and some strains of *Staphylococcus* can be liberated after disintegration of the cell-wall with preparations of lysozyme.

THE NATURE OF ENZYMES

Such studies often lead to knowledge concerning the nature of the enzymes concerned and it has been found that although all enzymes have the properties of proteins, many of them consist of two parts, one protein in nature and the other, called the prosthetic group, of a simpler non-protein nature. Prosthetic groups can often be detached from the protein moiety—in which case the enzymatic activity ceases—and their structure determined. The link between the prosthetic

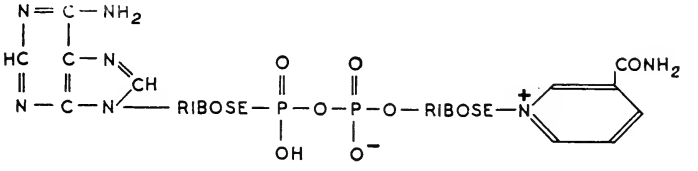
[1] Booth and Green, *Biochem. J.*, 1938, **32**, 855.
[2] Werkman *et al*, *J. Bact.*, 1945, **49**, 595.
[3] Curran and Evans, *J. Bact.*, 1942, **43**, 125.

group and the protein varies in strength so that some prosthetic groups are firmly fixed to the protein whilst others are in such a loose combination that they may wander from one protein molecule to another. In this second case the non-protein moiety is called a " coenzyme." There is still some doubt whether there is any difference between prosthetic groups and coenzymes other than in the strength of the link with the protein, or whether there is a fundamental difference in that the prosthetic group is an integral part of the enzyme structure while the coenzyme acts as a separate carrier of hydrogen ions, etc., from one enzyme to another. This controversial point is outside the scope of the present discussion so we shall discuss all prosthetic groups and coenzymes under one heading.

PROSTHETIC GROUPS

The prosthetic groups (and coenzymes) which have been identified up to the present are:

Adenine-nicotinamide-dinucleotide (Coenzyme I)

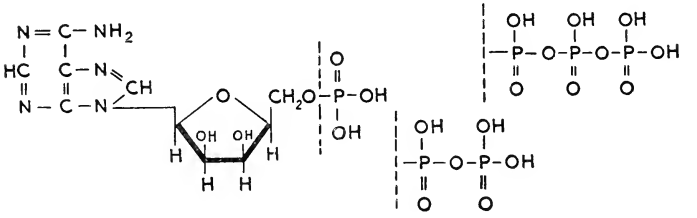


This acts as the coenzyme for certain dehydrogenases and these enzymes display a specificity towards coenzyme I as the hydrogen acceptor in the same way as they display specificity towards their substrate as hydrogen donator. The molecule appears to act as a carrier of hydrogen by alternate reduction and oxidation of the nicotinamide group. By accepting hydrogen from one dehydrogenase system and transferring it to another dehydrogenase system working in reverse, the coenzyme acts as a hydrogen carrier between what are called " coenzyme-linked-dehydrogenase systems " (see Chap. VII).

Coenzyme II

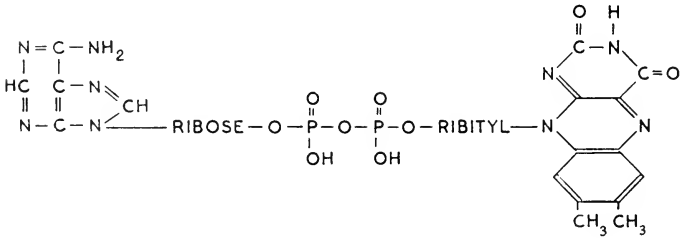
Coenzyme II has a structure similar to that of coenzyme I, with an additional phosphate group in the molecule; the position of the third phosphate in the molecule is not yet certain. The coenzyme acts as hydrogen acceptor towards certain dehydrogenase systems in a manner analogous to that of coenzyme I.

Adenylic acid, Adenosine-di-phosphate, Adenosine-tri-phosphate



Adenosine-tri-phosphate (ATP) acts as a donator of phosphate in phosphorylation reactions such as those that occur in fermentation cycles (see Chap. VII). The phosphate is linked in ATP by an energy-rich bond so that its rupture gives rise to the liberation of energy. Adenylic acid and adenosine-di-phosphate can act as phosphate acceptors, being synthesised to ATP. Since the glycolysis cycle depends upon phosphorylation and dephosphorylation (see Chap. VII) ATP and adenylic acid act as coenzymes in the cycle.

Adenine-riboflavin-dinucleotide, Flavine-adenine-dinucleotide

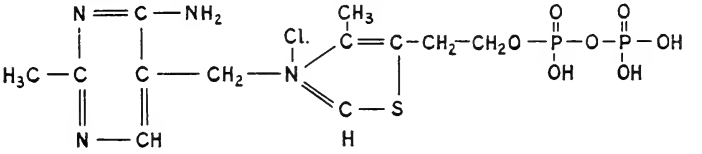


This forms the prosthetic group of enzymes known as flavoproteins and again acts as hydrogen carrier by alternate reduction and oxidation of the double bond in the isoalloxazine ring.

Riboflavin-phosphate

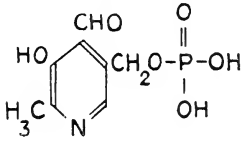
Consists of riboflavin with a single phosphate radicle; occurs as the prosthetic group of a flavoprotein enzyme known as cytochrome reductase (see below).

Thiamindiphosphate, Aneurindiphosphate, or Cocarboxylase

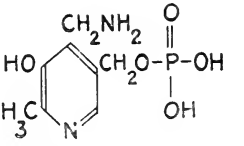


Thiamindiphosphate was first identified as the prosthetic group of yeast carboxylase, the enzyme which decarboxylates pyruvic acid to acetaldehyde. It is found in many tissues and bacteria which do not possess carboxylase and plays a part in many of the reactions involving pyruvic acid (see Chap. VII), such as oxidative decarboxylation. It also acts in some as yet undefined way in the oxidation of certain fatty acids.

Pyridoxal phosphate, Pyridoxamine phosphate



(*a*) Pyridoxal phosphate.

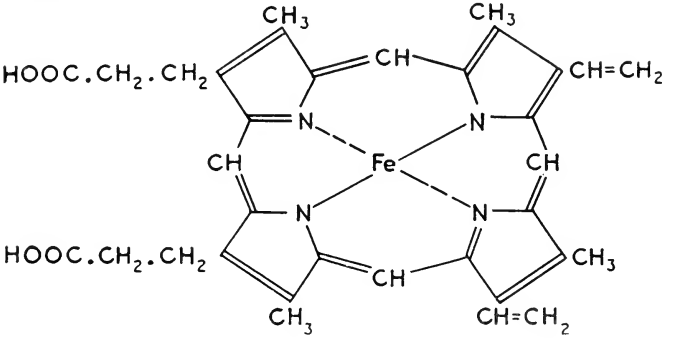


(*b*) Pyridoxamine phosphate.

Pyridoxal phosphate has the probable structure shown above, although the exact position of the phosphate group is not yet certain. It acts as the prosthetic group of the amino-acid decarboxylases (see Chap. IX) and of bacterial transaminase.

In the latter case it probably reacts with the $—NH_2$ group of an amino-acid and is converted to pyridoxamine phosphate, leaving the keto-acid corresponding to the amino-acid. Pyridoxamine phosphate can then react with a suitable keto-acid converting it to the corresponding amino-acid while being itself restored to the pyridoxal form.

Haematin



This forms the prosthetic group of haemoglobin and of enzymes such as catalase, peroxidase, and cytochrome oxidase. Side chains may differ in different enzymes, etc.

Metals

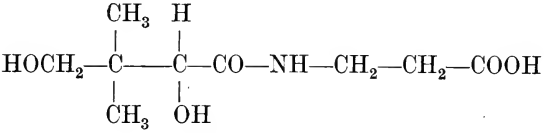
Some enzymes contain a metal in their structure, while others are activated by the presence of metal coenzymes. Thus phosphatase is activated by magnesium ions, while in other cases metals such as copper (polyphenol oxidase) or zinc (carbonic anhydrase) appear to be an essential part of the enzyme structure.

PROSTHETIC GROUPS OF INCOMPLETELY DETERMINED STRUCTURE

Coenzyme A

Lipmann, in the course of studies on the acetylation of sulphanilamide by pigeon liver, discovered that the acetylase involved possessed a prosthetic group which could not be

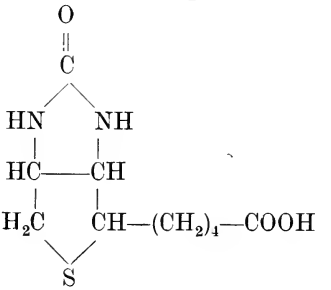
replaced by any known coenzyme. Preparations of the new coenzyme A, as it was called, were found to contain pantothenic acid and their activity could be correlated with their pantothenate content. The complete structure of coenzyme A has not yet been determined and the complete molecule is more complex than pantothenic acid alone. Coenzyme A is now known to act in many reactions involving acetic acid, or "acetyl phosphate," and is required by bacteria for the acetylation of choline. Recently it has been shown that a cell-free extract of *Esch. coli* will catalyse the condensation of acetyl phosphate and oxalacetic acid to give citric acid (see p. 153) and that the extract is activated by coenzyme A.



Pantothenic acid

Biocytin

The aspartic deaminase (see p. 162) of *Esch. coli* has been shown to require a coenzyme which contains biotin and is probably a compound of biotin and adenylic acid. The complete structure is not yet known. A crystalline preparation of biocytin, which is a compound of biotin present in yeast extracts, has now been made but it is not yet certain whether this is identical with the aspartic co-deaminase or not.



Biotin

NATURE OF ENZYME CATALYSIS

Knowledge of the nature and function of the prosthetic groups becomes of importance in understanding the nutrition of exacting bacteria (see Chap. V). Several enzymes may have the same prosthetic group but, nevertheless, have different substrate specificities. It is probable that the prosthetic group plays an active part in the decomposition of the substrate, but that the protein moiety of the enzyme is responsible for the specificity towards the substrate. It is thought that a type of loose combination takes place between the substrate and the enzyme protein before catalysis occurs. For instance we often find that the enzyme action is dependent upon the presence, not only of the chemical group whose alteration is catalysed, but also of other groups in the substrate molecule. Lysine decarboxylase cannot catalyse the decarboxylation of lysine unless both α and ϵ $-NH_2$ groups of the lysine molecule $H_2N.CH_2.CH_2.CH_2.CH_2.CHNH_2.COOH$ are intact and unsubstituted. Further, the enzyme cannot attack the *dextro*-isomer of lysine nor can it attack L-ornithine, which differs from L-lysine in having one less C atom in the carbon chain. Before L-lysine decarboxylase can attack its substrate, this must possess:

1. an unsubstituted —COOH group,
2. an intact alpha $-NH_2$ group in the *laevo*- position,
3. an intact $-NH_2$ group in the terminal position,
4. the distance corresponding to $-CH_2.CH_2.CH_2.CH_2.CH-$ between the two amino-groups.

The —COOH and $-NH_2$ groups are chemically reactive or "polar" groups, and it is thought that a combination between these groups and corresponding groups on the surface of the enzyme protein must take place before the decarboxylation is catalysed. L-Lysine decarboxylase can thus be thought of as combining with the two amino-groups of lysine as a preliminary to the removal of the —COOH group. Substitution of $-CH_3$, etc., in either $-NH_2$ would

prevent the enzyme-substrate attachment, as would alteration of the carbon chain length between them as this would place the two $—NH_2$ groups at the wrong distance apart to orientate and attach to the combining groups of the enzyme surface. Such a substrate-enzyme relation would also explain optical specificity as it is obvious that the *laevo-* and *dextro-* forms of the substrate would not " fit " on to the same combining group structure. This hypothesis that the substrate and enzyme-surface have to fit or interlock in an exact position has given rise in the past to the analogy of a lock and key, in that the one must fit the other before any further action can occur.

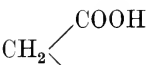
If the combining groups on the surface of the enzyme are almost, but not quite, in the right position to " fit " the polar groups of the substrate, then the resulting enzyme-substrate combination will introduce a strain into the structure of the substrate and so render it more unstable. Haldane has suggested that the properties of enzyme action can be explained by such enzyme-substrate combinations which result in the production of a strain in the substrate molecule and facilitate its chemical alteration.

COMPETITIVE INHIBITION

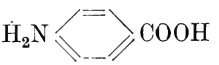
The substrate of an enzyme therefore is any substance which can combine reversibly with the right groups on the enzyme surface. The reversible nature of the combination is important, for if we can find a substance whose structure is such that it can combine with the combining-groups on the enzyme surface but which is not strained, altered, and released as is the true substrate, then this substance will remain on the surface of the enzyme, block the essential links, and so prevent the true substrate from combining. The net result of this is that the breakdown of the substrate is inhibited and such a substance is called a " competitive inhibitor." An example of a competitive inhibitor is malonic acid, $HOOC.CH_2.COOH$, which combines with succinic dehydrogenase and inhibits the dehydrogenation of the true substrate,

succinic acid, presumably by reason of the similarity of the structures—especially the spatial relations of the two polar groups in the —COOH groups—of substrate and inhibitor. It is characteristic of this type of inhibition that the degree of inhibition depends upon the relative proportions of substrate and inhibitor present. It will be seen later that the bacteriostatic action of the sulphonamide drugs has been explained in terms of their competitive inhibition of the essential metabolism of structurally similar *p*-amino-benzoic acid.

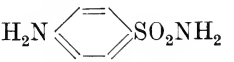
Succinic acid



Malonic acid



p-Amino-benzoic acid



Sulphanilamide

TYPES OF ENZYMES IN BACTERIA

Enzymes are classified according to the reactions which they catalyse. It will have been obvious from the above that an enzyme catalysing a dehydrogenation is called a "dehydrogenase," one catalysing a decarboxylation a "decarboxylase," etc., the name of the substrate usually being specified as well. Bacteria possess a great variety of enzymes and these will be discussed briefly under the general headings set out in Chap. I as indicating the basic reactions carried out by bacteria (pp. 5-6).

1, 2. Reduction and Oxidation

Most biological oxidations are of the nature $AH_2 + B = A + BH_2$, where the substance AH_2 is oxidised to A and the substance B reduced to BH_2. The oxidation of AH_2 is catalysed by a dehydrogenase specific for that substrate. Many dehydrogenases have been obtained in a cell-free state from yeasts and animal tissues, but until recently the problem

of rupturing the bacterial cell-wall has made the study of cell-free bacterial enzymes a matter of considerable difficulty. However, using the recently invented methods of breaking bacterial cells by grinding with glass particles, shaking with minute glass beads, exposure to supersonic vibration, etc. (see p. 30), it has been possible to obtain a number of dehydrogenases in a cell-free state and they do not appear to differ significantly from their counterparts in other cells. Amongst the enzymes isolated from *Esch. coli* we have those which will specifically dehydrogenate formic acid to CO_2, L-malic acid to oxalacetic acid, ethyl alcohol to acetaldehyde, triosephosphate to phosphoglyceric acid, succinic acid to fumaric acid, etc. The action of the dehydrogenase can be written

$$AH_2 = A + 2H,$$

and the dehydrogenation cannot take place until a hydrogen acceptor B is available:

$$AH_2 + B = A + BH_2.$$

The dehydrogenases are specific towards the hydrogen acceptor as well as towards the substrate. In some cases the hydrogen acceptor is oxygen, in which case the reaction is either

$$AH_2 + O_2 = A + H_2O_2 \quad \text{or} \quad AH_2 + O = A + H_2O,$$

but we find in practice that only relatively few dehydrogenases can utilise oxygen as hydrogen acceptor. An example of such an enzyme is the D-amino-acid oxidase of animal tissues which reacts

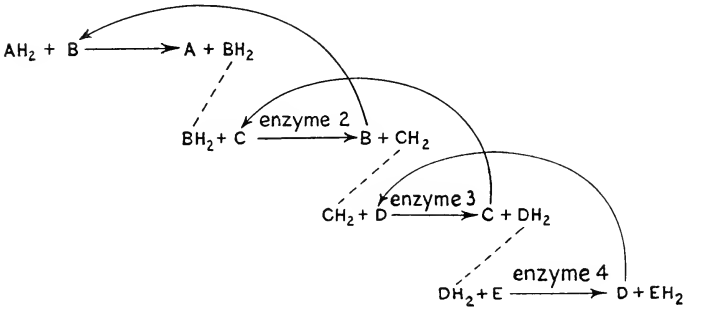
$$R.CHNH_2.COOH + O \longrightarrow R.C{:}NH.COOH + H_2O,$$

followed by spontaneous hydrolysis of the imino-acid to the corresponding keto-acid with liberation of ammonia.

The majority of the dehydrogenases effect a transfer of hydrogen from the substrate to an intermediate carrier represented by B in the above equation. The investigation of such oxidation-reduction reactions has been carried out

mostly with animal tissues or yeast-cells, so it will be best to summarise the findings for enzymes from these cells and then outline their application to bacteria. The general findings are summarised in Table III.

In animal tissues and yeasts the transfer of hydrogen from substrate to oxygen passes through several intermediate oxido-reduction reactions with oxygen as the final hydrogen acceptor in the chain.



The ultimate carrier (D) is a haematin-protein called cytochrome which is capable of alternate oxidation and reduction. Reduced cytochrome becomes oxidised again by the action of the enzyme cytochrome oxidase. Oxidised cytochrome can be reduced directly by the action of certain dehydrogenases transferring hydrogen from their substrates to cytochrome. The chain in this case (substrate type S_2, Table III) can be represented:

$$AH_2 + \text{Cytochrome} \xrightarrow[\text{dehydrogenase}]{AH_2} A + \text{Reduced cytochrome}$$

$$\text{Reduced cytochrome} + O \xrightarrow[\text{Oxidase}]{\text{Cytochrome}} \text{Cytochrome} + H_2O$$

The cytochrome is thus alternatively reduced by the action of the dehydrogenase and oxidised by the action of cytochrome

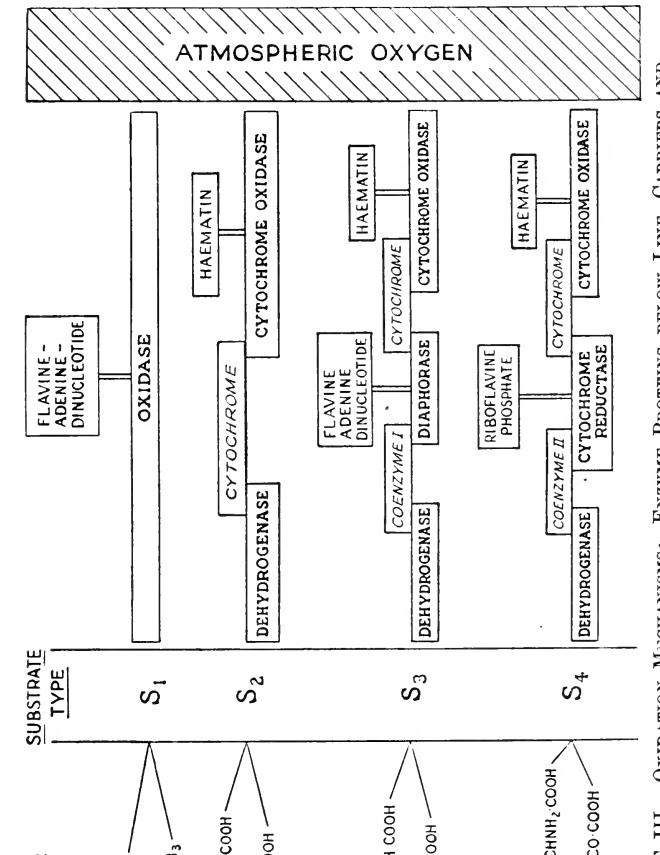
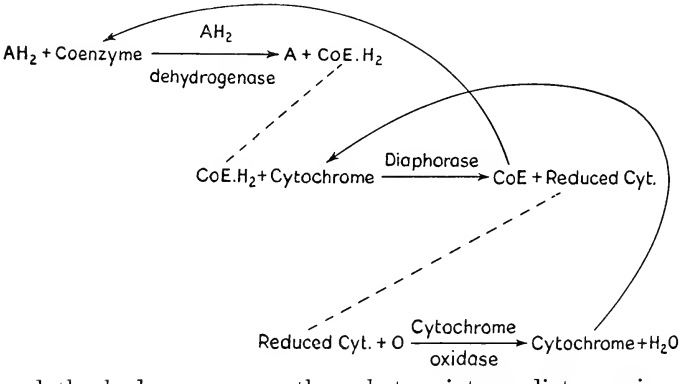


TABLE III. Oxidation Mechanisms: Enzyme Proteins below Line, Carriers and Prosthetic Groups above.

oxidase, the net result being the oxidation of the dehydrogenase substrate.

Enzymes which transfer hydrogen direct from their substrate to oxygen are called " oxidases "; the next simplest system is the direct cytochrome system described above. The majority of dehydrogenases are unable to transfer hydrogen from their substrate to cytochrome without the intermediation of a further carrier in the form of a coenzyme. Dehydrogenases of this third type can be looked upon as enzymes with prosthetic groups so loosely attached that the latter can become detached from the protein and act as hydrogen carriers between one enzyme system and the next. In this case the action of the dehydrogenase is to transfer hydrogen from the substrate to the coenzyme. The formulae of coenzymes I and II have been given on p. 31. These substances act as hydrogen acceptors by reduction of one of the double bonds in the pyridine ring of nicotinamide. The reduced coenzyme is not autoxidisable but acts as the specific substrate for a coenzyme dehydrogenase (" diaphorase " for coenzyme I, " cytochrome reductase " for coenzyme II) which transfers the hydrogen to cytochrome. In this case the reaction chain is (substrate types S_3 and S_4, Table III):



and the hydrogen passes through two intermediate carriers before linking with oxygen.

In this manner oxidations involving large changes of energy are split up into a number of steps, each involving smaller changes of energy, and each catalysed by a specific enzyme. There are yet more complex respiratory systems involving further carriers in the chain between initial substrate and oxygen, but we need not discuss these here as our knowledge of bacterial oxidation has not yet progressed beyond the stages so far outlined.

In order of increasing complexity then we have:

(*a*) OXIDASE SYSTEMS: substrate type S_1, Table III.

In animal tissues we have oxidases attacking D-amino-acids, amines, uric acid, etc., but few oxidases have so far been identified in bacteria. Some organisms oxidise amines to the corresponding aldehydes, but no analysis of the enzyme systems involved has yet been made. Aerobic organisms and those possessing cytochrome (Table IV) possess cytochrome oxidase. An L-amino-acid oxidase has been found in *Proteus vulgaris* and obtained in a cell-free condition by supersonic disintegration of the cells: it carries out the reaction

$$R.CHNH_2.COOH + O \longrightarrow R.CO.COOH + NH_3$$

in which, presumably, the first step is a dehydrogenation to the unstable imino-acid

$$R.CHNH_2.COOH - 2H \longrightarrow R.C{:}NH.COOH.$$

The oxidation of their substrates by certain mammalian oxidases gives rise to the production of H_2O_2, but this L-amino-acid oxidase of *Pr. vulgaris* is said not to produce peroxide and no evidence has been presented concerning the nature of the protein, whether it has a flavine prosthetic group or not.

Hydrogen peroxide is highly toxic to living cells and many organisms possess an enzyme, catalase, which destroys peroxide by breaking it down to water with the liberation of oxygen

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2.$$

Catalase is a haematin-enzyme and recently it has been shown

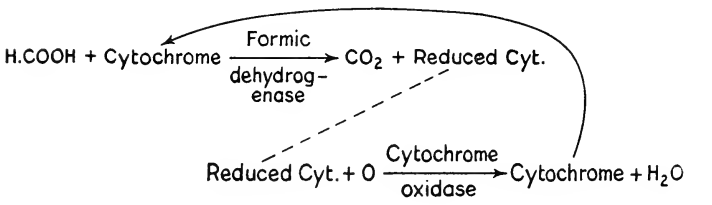
that catalase can also catalyse the oxidation of certain substrates utilising H_2O_2 as hydrogen acceptor,

$$AH_2 + H_2O_2 \xrightarrow{\text{Catalase}} A + 2H_2O.$$

Such a reaction is said to be a " coupled oxidation " and the result is again the removal of hydrogen peroxide.

(*b*) Cytochrome systems: substrate type S_2, Table III.

The formic dehydrogenase of *Esch. coli* belongs to this class.



The cytochrome system of animal- and yeast-cells consists of at least three components which are distinguished by the position of their absorption bands in the visual spectrum; these components are known as cytochromes a, b, and c. Cytochrome b is slowly autoxidisable but cytochromes a and c cannot react with oxygen in the absence of cytochrome oxidase. The cytochrome systems of bacteria differ from those of animal- and yeast-cells in that bacteria may have several or none of the components. *Esch. coli* has one component only and this has absorption bands corresponding to those of cytochrome b, but differs in that it is not autoxidisable; it is usually referred to as cytochrome b_1. The distribution of the cytochrome components as identified by their absorption bands in various bacteria is given in Table IV: the letters a, b, and c are given as for animal tissues, but it is by no means certain that these bacterial cytochromes are identical with those in other tissues.

It can be seen from the table that certain species possess no cytochrome components, and consequently cannot carry

out oxidation mechanisms of the cytochrome type. It is possible that some other carrier might take the place of cytochrome but the only one of these organisms which is known to produce a pigment which can be reversibly oxidised and reduced, other than cytochrome, is *Ps. pyocyanea* (*Ps. aeruginosa*). This elaborates a blue pigment, pyocyanine, which is capable of acting as a hydrogen-carrier with certain dehydrogenase systems when tested *in vitro*. This organism has, however, a full complement of cytochrome components.

TABLE IV

DISTRIBUTION OF CYTOCHROME COMPONENTS IN BACTERIA

Strict aerobes:			
Mycobacterium tuberculosis	a	b	c
Azotobacter chroococcum	—	b	c
Facultative anaerobes:			
Ps. pyocyanea	a	b	c
Staph. aureus	a	b	—
Esch. coli	—	b	—
Pneumococcus	a	b	—
S. faecalis	—	—	—
Lactobacillus acidophilus	—	—	—
Strict anaerobes:			
Cl. tetanum	—	—	—
Cl. welchii	—	—	—

The organisms devoid of cytochrome are either strict anaerobes such as the Clostridia, or microaerophilic such as the Streptococci or Lactobacilli; this suggests that the absence of cytochrome components leads to the inability of these cells to utilise oxygen. Isolated dehydrogenase systems can be made to react *in vitro* by replacing cytochrome with certain "redox" indicators such as methylene blue or cresyl blue; the dehydrogenase catalyses the transference of hydrogen from substrate to the dye, and the reduced dye is autoxidisable. When cytochrome is replaced in this way the final product of the oxidation is H_2O_2 and not H_2O, so that the complete

reaction is $AH_2 + O_2 = A + H_2O_2$, resembling some of the oxidase mechanisms discussed above. The distribution of catalase is not universal amongst bacterial species, and those organisms which are devoid of cytochrome are often devoid also of catalase. It has been suggested that organisms such as the Clostridia, the Streptococci, etc., owe their sensitivity to the presence of oxygen to the fact that, being devoid of catalase, they become poisoned by the formation of H_2O_2 under aerobic conditions. The amounts of H_2O_2 which would be formed and which might be toxic are so small as to be beyond present methods of detection, and this point has yet to be satisfactorily investigated. It is suggestive, however, that Pneumococci can be protected in the presence of air by pyruvic acid, and it is known that pyruvic acid and H_2O_2 react together chemically in such a way as to destroy the H_2O_2.

(*c*) COENZYME SYSTEMS: substrate types S_3 and S_4, Table III. In *Esch. coli* we have coenzyme systems which are apparently identical with those in animal- and yeast-cells; thus L-malic acid dehydrogenase:

$$\begin{array}{l} CH_2.COOH \\ | \\ CHOH.COOH \\ \text{L-Malic acid} \end{array} + \text{Coenzyme I} \xrightleftharpoons[\text{dehydrogenase}]{\text{Malic}} \begin{array}{l} CH_2.COOH \\ | \\ CO.COOH \\ \text{Oxalacetic acid} \end{array} + \text{Reduced coenzyme I}$$

and alcohol dehydrogenase:

$$CH_3.CH_2OH + \text{Coenzyme I} \xrightleftharpoons[\text{dehydrogenase}]{\text{Alcohol}} CH_3.CHO + \text{Reduced coenzyme I.}$$

The coenzyme I of *Esch. coli* has never been isolated in sufficient quantity and purity for its chemical structure to be determined, but we know that (1) the alcohol and L-malic dehydrogenases of *Esch. coli* will not reduce cytochrome or methylene blue in the absence of a coenzyme which can be

replaced *in vitro* by coenzyme I isolated from yeast, and (2) the malic dehydrogenase of animal tissues is likewise inactive in the absence of a coenzyme which can be supplied by the autogenous coenzyme I or by partially purified extracts of *Esch. coli*. Quantitative studies of these relationships leave no doubt but that the coenzyme I of yeast and animal tissues and the L-malic codehydrogenase of *Esch. coli* are identical.

Coenzyme II systems also exist in bacteria; for example, the L glutamic acid dehydrogenase of *Esch. coli*:

$$\begin{array}{l} CH_2.CH_2.COOH \\ | \\ CHNH_2.COOH \end{array} + \text{Coenzyme II} \rightleftharpoons \begin{array}{l} CH_2.CH_2.COOH \\ | \\ C : NH.COOH, \end{array} + \text{Reduced coenzyme II}$$

and in this case the dehydrogenase cannot be activated by coenzyme I, although the corresponding L-glutamic acid dehydrogenase of animal tissues is specific for coenzyme I. These coenzyme specificities are worked out with isolated enzymes *in vitro*, and it is probable that the intact bacterial cell can interconvert coenzymes I and II. The reduced coenzymes cannot react with cytochrome without the intervention of the coenzyme dehydrogenases; little work has been done on the coenzyme dehydrogenases of bacteria, and there is no evidence that these are any different from the similar enzymes of other cells.

LINKED OXIDATION-REDUCTION REACTIONS: So far in this section we have discussed the oxidation of various substrates. Many of the dehydrogenases are reversible and can carry out the general reaction

$$A + 2H \longrightarrow AH_2$$

in the presence of a suitable hydrogen donator. Reduced coenzyme can act as H-donator in this way and so can act as H-carrier between one dehydrogenase and another.

$$AH_2 + Co \longrightarrow A + CoH_2 - - - - CoH_2 + B \longrightarrow BH_2 + Co.$$

In this case AH_2 has been oxidised anaerobically by the

reduction of B. Several such "coenzyme-linked" oxido-reductions have been demonstrated in fermentation reactions (Chap. VII). In the Clostridia we find an oxido-reduction occurring between two amino-acids:

$$\begin{matrix} R.CHNH_2.COOH \\ \\ X.CHNH_2.COOH \end{matrix} + H_2O \longrightarrow \begin{matrix} R.CO.COOH \\ \\ X.CH_2.COOH. \end{matrix} + 2NH_3$$

The enzymes in this reaction have not as yet been investigated in detail, although it is possible to demonstrate the presence of the specific dehydrogenases for $R.CHNH_2.COOH$ and $X.CHNH_2.COOH$ (Chap. IX). The Methanobacteria carry out an interesting oxido-reduction reaction, in which alcohols are oxidised anaerobically with CO_2 acting as the H-acceptor, and being reduced to methane (p. 154).

$$2CH_3.CH_2OH + CO_2 \longrightarrow 2CH_3.COOH + CH_4.$$

HYDROGENASE: Many bacteria are able to activate molecular hydrogen as H-donator by the possession of a potent enzyme, hydrogenase, which catalyses the reaction:

$$H_2 \overset{\text{Hydrogenase}}{\rightleftharpoons} 2H.$$

The presence of this enzyme can be demonstrated in *Esch. coli* by shaking a suspension of cells with methylene blue in the presence of gaseous hydrogen when the dye becomes reduced and decolourised. Boiling of the organisms or replacing the hydrogen by any other gas stops the reduction. In this reaction methylene blue acts as H-acceptor for hydrogen made available by hydrogenase and, similarly, other suitable H-acceptors can be reduced in the presence of hydrogen. *Esch. coli* possesses an enzyme, nitratase, which activates nitrate as H-acceptor, so in the presence of the cells and hydrogen, nitrate is reduced to nitrite:

$$HNO_3 + 2H \longrightarrow HNO_2 + H_2O.$$

The reduction does not stop at this point but continues all

the way to ammonia with the probable intermediate formation of hydroxylamine,

$$HNO_2 + 4H \longrightarrow NH_2OH + H_2O$$

$$NH_2OH + 2H \longrightarrow NH_3 + H_2O,$$

the sum total of the reduction being represented,

$$HNO_3 + 4H_2 \longrightarrow NH_3 + 3H_2O.$$

3. Dehydration

The removal of H_2O from a substrate molecule is comparatively rare within our knowledge. In those cases where such a reaction is the result of the action of a single enzyme, that enzyme is called a dehydrase. Such an enzyme has been postulated to explain the breakdown of L-serine to pyruvic acid by *Esch. coli*:

$$\begin{array}{l} CH_2OH \\ | \\ CHNH_2{-}H_2O \\ | \\ COOH \end{array} \xrightarrow[\text{dehydrase}]{\text{Serine}} \begin{array}{l} CH_2 \\ \| \\ C.NH_2 \\ | \\ COOH \end{array} \rightleftharpoons \begin{array}{l} CH_3 \\ | \\ C{:}NH \\ | \\ COOH \end{array} \overset{H_2O}{\rightleftharpoons} \begin{array}{l} CH_3 \\ | \\ CO + NH_3 \\ | \\ COOH \end{array}$$

The first step in the postulated breakdown being a dehydration of serine to the corresponding imino-acid which hydrolyses spontaneously to pyruvic acid. A very similar reaction takes place with cysteine, in which the first step is the removal of H_2S (instead of H_2O) from the molecule, after which the course of the breakdown is the same (Chap. IX).

4. Hydrolysis

Hydrolytic enzymes are responsible for the processes known as digestion, whereby proteins are broken down to amino-acids, fats to fatty acids and glycerol, complex polysaccharides to simpler polysaccharides and monosaccharides, etc.

Proteolytic enzymes of many types are known, and have in common the power to hydrolyse the linkage —CO—NH—, splitting the peptide containing that linkage into two substances, one with a free —COOH and the other with a free

—NH_2 group arising from the broken peptide link. Proteolytic enzymes differ in specificity towards the chemical groups on either side of the —CO—NH— link, towards the length of the peptide chain they can attack, and towards the nature of the terminal groups of that chain. Some proteases are able to attack large protein molecules in a native state, others can attack only after the protein has been denatured, others can attack relatively short polypeptide chains, and others are specific for peptides of two, three, or four amino-acid residues of definite structure. Some peptidases display specificity towards the nature of the particular amino-acids on either side of the peptide link to be hydrolysed, and much of our knowledge concerning stereo-specificity and the " lock and key " nature of enzyme action has been deduced from studies of particular peptidases and the structure of the peptides they can attack. The proteases and peptidases of animal tissues have been studied in considerable detail, but our knowledge of the proteolytic enzymes of bacteria is so far meagre.

In order to digest large protein molecules which cannot pass through the cell-wall, bacteria excrete extracellular proteases into the surrounding medium, and the power to do this seems to be restricted to comparatively few species. The majority of bacteria can hydrolyse the simpler molecules of peptone and polypeptides, but with a few notable exceptions no detailed studies or separation of the enzymes involved has been undertaken. Some organisms excrete an enzyme which specifically hydrolyses gelatine and consequently these organisms bring about a liquefaction of gelatine media on which they are grown—this property is used as a diagnostic test in systematic bacteriology—but the power to form gelatinase is not necessarily accompanied by the ability to excrete proteases.

The ability to hydrolyse polysaccharides again involves the excretion of extracellular enzymes, and the ability to do this is subject to the same degree of species variation as any of the fermentation reactions used for characterisation tests in

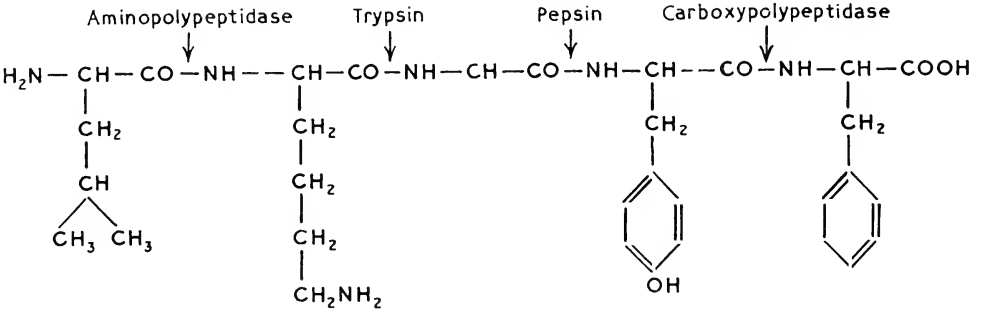


Diagram illustrating action of animal proteolytic enzymes.

Aminopeptidases attack peptide links adjacent to a free —NH_2 group in a leucyl residue.

Carboxypeptidases attack peptide links adjacent to a free —COOH group in a tyrosyl or phenylalanyl residue.

Trypsin attacks peptide links in the middle of a peptide chain but the link must possess lysyl or arginyl residues attached to the carbonyl side of the link.

Pepsin attacks peptide links in which tyrosyl or phenylalanyl residues are attached to the imino side of the link.

systematic bacteriology (Chap. II). The specificity of the polysaccharide-splitting enzymes depends upon the nature of the linkages between the sugar units of the polysaccharide chain. *Cl. welchii*, for example, cannot hydrolyse starch unless it is first grown in the presence of starch which then evokes the production of the enzyme. The organism grown in the presence of starch can also hydrolyse maltose and glycogen. Likewise, if the organism is grown in the presence of maltose, then it gains the power to hydrolyse starch and glycogen. Consequently the enzyme or enzymes necessary for the hydrolysis of starch, glycogen, or maltose cannot be produced unless growth takes place in the presence of any one of these carbohydrates. Growth in glucose does not produce the enzyme. Full investigation has not been made, but the assumption is that we are dealing with the production of an adaptive enzyme (Chap. IV), specific for the hydrolysis of the maltose linkage. The breakdown of starch by *Cl. acetobutylicum* is accomplished by the production of two enzymes; first, an amylase which hydrolyses the starch to maltose, and, secondly, a maltase which completes the hydrolysis of maltose to glucose. Cellulose is attacked by a variety of organisms normally found in the rumen and on plant tissues. *Cellulobacillus myxogenes* produces two enzymes responsible for the hydrolysis of cellulose, the first, cellulase, hydrolyses cellulose (see p. 120) to cellobiose, and the second, cellobiase, completes the hydrolysis of cellobiose to glucose.

5. Deamination

The removal of $—NH_2$ from the molecule of an amino-acid, amine, etc., is seldom achieved in a single step. We have already had two examples of deamination occurring in two steps: the amino-acid oxidase of *Pr. vulgaris*, and the L-glutamic acid deaminase of *Esch. coli*, where the first step is a dehydrogenation of the amino-acid to the corresponding imino-acid which then undergoes spontaneous hydrolysis to the corresponding keto-acid liberating ammonia:

$$\begin{array}{l} R.CHNH_2.COOH \rightleftharpoons R.C:NH.COOH + 2H (\rightarrow \text{coenzyme}) \\ \qquad\qquad\qquad\qquad\quad \uparrow\downarrow H_2O \\ \qquad\qquad\qquad\qquad R.CO.COOH + NH_3. \end{array}$$

Deamination of amino-acids can also take place by reduction, desaturation, or hydrolysis (Chap. IX), but in the majority of these cases the intermediate steps, if any, are not known.

6. Decarboxylation

The removal of CO_2 from the molecule has been observed with two types of compound, keto-acids and amino-acids. In yeasts α-keto-acids are decarboxylated by the enzyme carboxylase which has been isolated in a cell-free condition and studied in a highly purified state. The enzyme consists of a protein and a loosely attached coenzyme or prosthetic group identified as thiamindiphosphate (see p. 33). Carboxylase attacks α-keto-acids, decarboxylating them to the corresponding aldehydes,

$$R.CO.COOH \xrightarrow{\text{Carboxylase}} R.CHO + CO_2.$$

Pyruvic acid, $CH_3.CO.COOH$, is attacked more rapidly than other acids of this group and, in general, the longer the carbon chain of the R group, the slower the rate of attack by the enzyme. Although thiamindiphosphate would seem to bear the same relation to carboxylase that coenzyme I does to L-malic dehydrogenase, we have as yet no definite knowledge of the way in which it functions in the decarboxylation of keto-acids. Carboxylase itself has not been found in the enzyme constitution of organisms such as *Esch. coli*, and it is possible that it does not enter into bacterial metabolism. The breakdown of pyruvic acid by bacteria is not by simple decarboxylation to acetaldehyde, but involves other mechanisms which also, however, require the presence of thiamindiphosphate (see Chap. VII).

Yeast carboxylase is specific for the decarboxylation of α-keto-acids, but in some bacteria we find enzymes which

remove CO_2 from other keto-acids. *Azotobacter* and *Micrococcus lysodeikticus* contain an enzyme which decarboxylates oxalacetic acid to pyruvic acid:

$$HOOC.CH_2.CO.COOH \xrightarrow[\text{decarboxylase}]{\text{Oxalacetic}} CH_3.CO.COOH + CO_2.$$

Cl. acetobutylicum possesses an enzyme which decarboxylates acetoacetic acid to acetone:

$$CH_3.CO.CH_2.COOH \xrightarrow[\text{decarboxylase}]{\text{Acetoacetic}} CH_3.CO.CH_3 + CO_2.$$

These enzymes have all been studied in a cell-free state and do not appear to involve thiamindiphosphate.

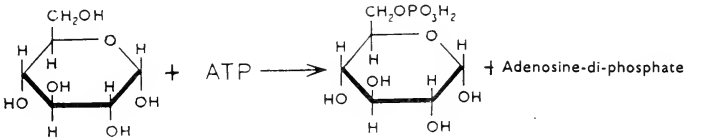
Some bacteria also carry out a decarboxylation of certain amino-acids to the corresponding amines.

$$R.CHNH_2.COOH \longrightarrow R.CH_2.NH_2 + CO_2.$$

The amino-acid decarboxylases are specific for a single amino-acid and, so far, enzymes have been isolated in a cell-free state which are specific for the natural isomers of lysine, arginine, histidine, ornithine, tyrosine, and glutamic acid. All these amino-acids have a polar group other than the α-NH_2 and the 1-COOH groups, and it has been found that substitution of the third polar group, *i.e.* the second $-NH_2$ group in lysine, ornithine, or arginine, the —OH in tyrosine, or the second —COOH group in glutamic acid, results in complete inhibition of the decarboxylation. This suggests that the enzyme and substrate must combine through at least two polar groups—other than the —COOH attacked—before decarboxylation can occur. The product of the decarboxylation is the corresponding amine or, in the case of glutamic acid, γ-amino-butyric acid. The decarboxylases of lysine, arginine, ornithine, tyrosine, and glutamic acid consist of a protein portion and a prosthetic group which can be replaced *in vitro* by pyridoxal phosphate (see p. 33).

7. Phosphorylation and dephosphorylation

The anaerobic breakdown of carbohydrate in yeast and in muscle involves the initial phosphorylation of the carbohydrate by a series of reactions involving adenosine-tri-phosphate and inorganic phosphate. The phosphorylated compounds then undergo a series of changes resulting in the formation of phosphopyruvic acid which is then dephosphorylated before the final stages of fermentation take place. The fermentation of glucose by *Esch. coli* and related organisms has now been investigated in considerable detail (Chap. VII), and appears to involve the same basic cycle of reactions as those occurring in yeast fermentation, so that phosphorylation of glucose to hexosediphosphate precedes breakdown to simpler molecules. The intermediate stages of the phosphorylation and the enzymes involved have not yet been worked out with bacteria, but it is highly probable that the first step is a transfer of phosphate from adenosine-tri-phosphate to the 6-position of glucose by the enzyme Hexokinase:



Many other bacterial fermentations will occur only in the presence of phosphate and are accompanied by an uptake of phosphate from the medium by the fermenting cells. The changes have seldom been investigated in detail and it is not possible to say whether phosphorylation is an invariable step in anaerobic carbohydrate breakdown. Fermentation of carbohydrate represents one of the main sources of energy for anaerobic existence. In those cases which have been worked out in detail, it appears that the incorporation of phosphate at low energy levels into organic compounds, followed by its removal at a later stage of the fermentation process from compounds in which the phosphate bond has become

" energy-rich," is the source of this utilisable energy (see Chap. VII). Considerable evidence is now accumulating that the energy obtained from oxidation processes also arises from the formation and breaking of energy-rich phosphate bonds; thus the oxidation of pyruvic acid by pyruvic oxidase of *Lactobacillus delbreuckii* is accomplished only in the presence of phosphate and involves the formation of acetyl phosphate as the first stage in the reaction:

$$CH_3.CO.COOH + H_3PO_4 + O = CH_3.COOPO_3H_2 + CO_2 + H_2O.$$

Phosphorylation may occur in many reactions other than those involved in fermentation and oxidation. Examples of phosphorylated intermediates are still being discovered in many metabolic changes as the biochemistry of living tissues is further probed.

General

The metabolism of a cell consists of many chemical changes catalysed by various enzyme systems. The biochemist has concerned himself with the isolation of these enzyme systems in an endeavour to identify the steps by which the changes occur and to interpret the mechanism of these changes in terms of the chemistry of the enzyme molecules. The systems outlined in this chapter summarise the types of enzymes that have been discovered in the course of these studies, but it must be realised that the metabolism of the intact cell is far more complicated than any of the separate reactions which can be studied *in vitro,* as we have not only the interplay of the various enzymes on each other and on the reactions catalysed by each other, but also the effect of environmental conditions on the formation of the enzymes themselves. We shall proceed to the discussion of this aspect of bacterial metabolism in the next chapter.

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CHAPTER IV

THE FORMATION OF ENZYMES IN BACTERIA

The manifold chemical activities of bacteria are catalysed by enzymes formed within the bacterial cell. Some bacterial species can exist under widely different chemical and physical environments, and require different types of enzymes in order to deal with the differing external conditions. *Esch. coli* utilises different enzymes for anaerobic existence from those utilised for aerobic existence, and needs different neutralisation mechanisms when growth takes place in an alkaline medium from those required when growth takes place in an acid medium. We find that an organism does not possess all the enzymes necessary for dealing with all possible environments at any one time, but that the actual enzymic constitution, as opposed to the potential enzymic constitution, is determined to a large extent by the external conditions holding during the formation of the individual cell. Consequently the cell grown aerobically is equipped with the mechanisms for oxidative metabolism, while the cell grown anaerobically is deficient in those mechanisms which can be utilised only under aerobic conditions but possesses highly developed anaerobic mechanisms. The actual enzymic constitution of a cell of a given species may thus vary widely. The identification of bacterial genera and species is based upon certain biochemical tests, but these are always carried out under standardised growth conditions and represent cross-sections of the potential enzymic constitution of the organism concerned. If an organism can ferment sucrose, then the fermentation will occur under the test conditions of growth in a fully nutrient medium containing sucrose, but it does not follow that the same organism can ferment sucrose if it is first grown in a medium free from sucrose or, say, nicotinamide.

THE POTENTIAL ENZYMIC CONSTITUTION

It is obvious that all organisms cannot form all enzymes. Otherwise any attempts at classification would fail. Although the enzymic constitution of an organism can and does undergo vast changes with alterations in the growth environment, there is still a limit to the changes that can occur and the enzymes that can be produced by any one organism. In higher organisms the enzymic constitution is controlled by the genetic composition of the cell. Genes are hypothetical units which determine the carry-over of characteristics from mother- to daughter-cell. In nucleate cells it is possible to observe changes in the form of the nucleus during division of the cell; the nucleus forms a skein instead of a solid body, the skein breaks up into short rod-like structures known as chromosomes and, in normal division, each chromosome divides into two before division, one of each pair passing into each daughter-cell; after the daughter-cell has split off, the chromosomes join up again into a skein which then collapses to form the new nucleus. The genes occupy definite positions on the chromosomes and damage to a chromosome in a certain place will be accompanied by loss of the property associated with possession of the gene lying at that place. Chromosomes have not yet been clearly demonstrated in bacteria, although structures allied to chromosomes have been described in some of the filamentous organisms. However, the general behaviour of bacteria, the inheritance of enzymic properties from one cell to another, and the occurrence of "mutants" suggest that some form of genetic control of enzyme constitution occurs in these organisms.

Neurospora crassa, a mould which commonly occurs on bread, has certain characteristics concerning the arrangement of its spores which make it easy to study from a genetical point of view. It has been found that it is possible to alter the genetic constitution of the mould by irradiation with X-rays. When this is done an occasional "hit" is made by the radiation on the chromosome and the absorption of a quantum of energy

results in an alteration of the gene at the site of the "hit." It has been found experimentally that whenever a gene is so altered, there is a loss of one enzyme from the enzymic constitution. From this work, and from other investigations carried out with yeast cells, it can be concluded that the formation of an enzyme by a cell is controlled in the first place by the presence of the correct gene and that one gene controls one enzyme. When a gene is altered and the enzymic constitution of the resulting cell changes, the new cell is said to be a "mutant." Mutants arise spontaneously, and, as a result of many studies which have been carried out on micro-organisms, it appears that any given gene may alter and give rise to a mutant about once in every 10^5 to 10^9 generations. Consequently a spontaneous mutant is a very rare thing but when we are dealing with large populations, and in bacterial cultures we normally deal with populations of 10^8–10^9 cells/ml., it is probable that mutants will be present and any change in the environmental conditions which favour the growth of the mutant rather than that of the unaltered mother culture, will give rise to a progressive selective growth of mutant cells. Likewise if the environment is not suitable for growth of the mutant, then it will not multiply and will not exert any significant effect upon the properties of the culture as a whole.

Biologists working with moulds and higher organisms are accustomed to thinking in terms of genes and mutations, but it is only during recent years that the application of these terms to bacteriology has been investigated in detail. The presence of a heritable factor involved in enzyme control has been shown by some masterly studies by Avery and his colleagues on the conditions governing the formation of the polysaccharide capsule of the Pneumococcus (see Chap. VI). Pneumococci are divided by serological methods into a number of types, and type specificity is conferred by the chemical structure of the polysaccharide capsule of the organism. Thus the capsule of a Type III *Pneumococcus* is composed of a

polysaccharide of different chemical structure from the polysaccharide of Type II *Pneumococcus*. Capsulated pneumococci will give rise under certain conditions to the growth of non-capsulated or "Rough" strains. Avery and his colleagues have shown that a rough non-capsulated Type II *Pneumococcus* will grow as a capsulated Type III *Pneumococcus* (*i.e.* acquire the enzyme necessary for the synthesis of the Type III polysaccharide) if an extract of Type III organisms is added to the medium. Careful investigation of the nature of the "transforming principle" in the extract shows that it is a desoxyribonucleic acid and it is active in a dilution of 1 part in 6×10^8 parts of medium. Further, once the Type II organism has been transformed into a Type III organism, it then continues to grow as a Type III organism, even when grown in the absence of the desoxyribonucleic acid. In this case the potential enzymic constitution of the organism has been altered by the addition to the organism at a certain stage of a minute amount of the nucleic acid, and it is tempting to think that this is equivalent to adding a gene to the genetic make-up of the organism.

The studies, mentioned above, with *Neurospora* have shown that alteration of a gene will result in the loss of an enzyme—and, presumably, reconstitution of the gene will result in the reappearance of the enzyme. The enzyme may thus be lost, or gained, by spontaneous mutation or the process may be artificially accelerated by irradiation or by treatment with "mutagenic" substances such as mustard gas. This sort of phenomena is well known in bacterial chemistry. Perhaps the earliest case to be studied was that of *Escherichia coli mutabile*: this is a variant of *Esch. coli* which will not ferment lactose and when grown on lactose plates containing indicator, produces white colonies, indicating no acid formation from lactose. If, however, the incubation is continued, small red papillae appear on the white colonies, indicating that new cells are growing which have the ability to ferment the sugar. If the non-fermenting culture is serially subcultivated several times in lactose-containing medium, then the power to ferment

the sugar is slowly acquired in the course of subcultivation, and after several such *passages*, the culture behaves as though it were a normal lactose-fermenting *Esch. coli*. Detailed investigation of the individual cells in the cultures (by plating out a high dilution on lactose- and -indicator-plates) shows that all cultures contain a number of fermenting cells and a number of non-fermenting cells. The initial non-fermenting culture is found to contain, on the average, one fermenting " mutant " for every 10^5 non-fermenting cells. In the course of cultivation in the presence of lactose, the proportion of fermenting cells increases, since the medium will obviously favour the growth of these mutants but the final fermenting culture will still contain a small proportion of non-fermenting cells.

It is not certain what is the difference between the fermenting and non-fermenting cells. It is probable that the fermenting cells possess the enzyme lactase, whereas the non-fermenting cells are mutants which have lost this enzyme. However, one investigator has claimed that both types of cell possess lactase but the non-fermenting one has a cell-wall impermeable to the disaccharide. Whatever may be the true difference, it is clear that growth in a lactose medium results in selective growth of the mutant able to utilise the sugar.

In the next chapter we shall be considering organisms which have lost the ability to synthesise certain amino-acids. A simple example is that of *Eberthella typhosa* which, when freshly isolated, is unable to synthesise tryptophan. The primitive type is able to synthesise the amino-acid; in the course of multiplication, mutants arise and, in approximately 10^7 generations, a cell arises which has lost one of the enzymes involved in tryptophan synthesis; if the organism is growing in the tissues of a host it will find tryptophan supplied in the medium and the synthetic disability will therefore not impose any restriction on growth. The synthesis of a substance such as tryptophan involves the expenditure of energy; consequently if the organism can grow by the assimilation of preformed tryptophan its growth process will be energetically more efficient and, in the course of many generations, the

slightly more efficient growth of the non-synthesising mutant will have the result that it will outgrow the synthesising cells. In the course of evolution the primitive cell, capable of tryptophan synthesis, has been lost and now only arises as a "back mutant" from the tryptophan-requiring organism. Fildes has shown that such back mutants can be demonstrated in cultures of *Eberthella typhosa*, and growth in a tryptophan-free medium will selectively grow the mutants and so give rise to cultures of the organism which are capable of synthesising their own tryptophan.

The spontaneous production of mutants can become highly important in considerations of drug resistance. A chemotherapeutic drug such as sulphanilamide acts by inhibiting an essential enzyme process (see Chap. V). If a mutational alteration occurs which renders that particular enzymic process non-essential, then the resulting organism is no longer sensitive to the drug. One method of selecting such insensitive mutants is to culture the organism in the presence of an amount of drug which limits the growth of all the sensitive cells. Such a procedure may take place accidentally during the clinical treatment of a patient infected with the organism and the appearance of sulphonamide-resistant strains of organisms such as *Staph. aureus* is well known to medical scientists. Selection of the mutants may arise accidentally, as described, or as a result of deliberate cultivation in the laboratory. Resistance may arise in small steps or cells may rapidly become completely resistant to a drug. One of the main drawbacks to the clinical use of streptomycin is that many organisms acquire a complete resistance to it within a very short period of cultivation in its presence.

The *actual* enzymic constitution of a cell is that portion of its *potential* enzymic constitution that is selected by the conditions under which it has been grown. Amongst the factors controlling this selection we may list the following: (*a*) the chemical constitution of the medium, (*b*) the physicochemical conditions holding during growth, and (*c*) the "age" of the culture.

CHEMICAL CONSTITUTION OF THE MEDIUM

Presence of substrate

This subject has been studied in considerable detail by Karström, who found that the ability to ferment certain sugars is often acquired only if growth takes place in the presence of those sugars. Table V shows the variation of the fermentation abilities of *Betacoccus arabinosaceus* with the nature of the sugar present during growth. From the table we can see that this organism ferments glucose and sucrose whether these sugars are present in the growth medium or not, but the fermentation of galactose, maltose, lactose, and arabinose will take place only if growth has occurred in the presence of galactose, maltose, lactose, or arabinose respectively. Galactose can be fermented if growth has taken place in the presence of lactose, since growth in lactose results in the liberation of galactose from the lactose molecule.

TABLE V

RELATION OF FERMENTATIVE PROPERTIES TO NATURE OF THE SUGAR PRESENT DURING GROWTH (*Betacoccus arabinosaceus*)

Sugar in Growth Medium	Fermentation Occurs with					
	Glucose	Galactose	Sucrose	Maltose	Lactose	Arabinose
No sugar ...	+	0	+	0	0	0
Glucose ...	+	0	+	0	0	0
Galactose ...	+	+	+	0	0	0
Sucrose ...	+	0	+	0	0	0
Maltose ...	+	0	+	+	0	0
Lactose ...	+	+	+	0	+	0
Arabinose ...	+	0	+	0	0	+

Karström therefore divided bacterial enzymes into two classes:

1. ADAPTIVE ENZYMES, which are formed only when growth takes place in the presence of the specific substrate, *i.e.* are formed only when required.

2. CONSTITUTIVE ENZYMES, which are formed whether growth occurs in the presence or absence of the substrate.

The application of quantitative studies to enzymes has now shown that adaptive enzymes are usually formed to a small degree even when growth occurs in the absence of the substrate, and that the presence of the substrate during growth results in a marked stimulation of the enzyme formation. One explanation that has been put forward of this difference between the enzymes is that the adaptive enzymes are unstable in the absence of their substrate and consequently lose their activity if growth takes place in the absence of the substrate. It is important to realise that this distinction between the two classes of enzymes is an experimental one, as in normal existence an organism will but rarely meet, after growth has ceased, with substances with which it has not been in contact during growth. The adaptive nature of an enzyme can only be shown by taking an organism after growth has taken place in the absence of the substrate, placing it in contact with the substrate, and comparing its activity with that of an organism grown in the presence of the substrate.

Adaptation of this nature takes place rapidly, for if an organism can ferment galactose adaptively, or, in other words, if it has an adaptive galactozymase, then a single cultivation in the presence of galactose will be sufficient to evoke the enzyme to its full extent. A single subsequent cultivation in the absence of galactose will result in the loss (or marked decrease in the activity) of the enzyme. The difference from an experimental point of view between adaptation and selection of mutants is illustrated in Fig. 1.

We can summarise the position regarding the formation of an enzyme in the growing cell as follows: the capacity to form the enzyme depends upon the presence of the corresponding gene; if the gene is present, then the actual formation of the

enzyme may further depend upon the presence of the substrate. The mechanism of adaptation may be that the gene controls the formation of an inactive enzyme-precursor which is only activated by the presence of the substrate; alternatively, it may be that the enzyme is formed in the presence of the gene but is itself unstable in the absence of its substrate.

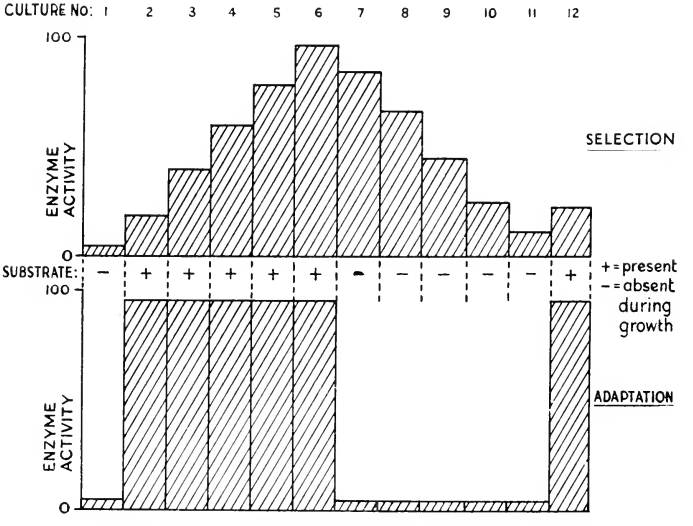

Fig. 1. Diagram to illustrate the difference between adaptation and selection processes in enzyme formation.

The presence of substances other than the substrate

Adaptation involves a relation between the organism, the enzyme, and the substrate, but sometimes substances other than the specific substrate may play a part in the formation or activity of an enzyme. The most marked example of this is found when fermentable carbohydrate is added to the medium. This problem has been studied chiefly with respect to the effect of the presence of glucose during growth on the production of enzymes concerned in the breakdown of proteins

and amino-acids, but the effect is by no means restricted to these enzymes alone. If we study the formation of a deaminase such as alanine deaminase, which carries out the reaction

$$CH_3.CHNH_2.COOH + O \xrightarrow[\text{deaminase}]{\text{Alanine}} CH_3.CO.COOH + NH_3,$$

in *Esch. coli* which has been grown in an amino-acid mixture with and without glucose, we find that the amount of deaminase produced in the absence of glucose is some twenty times greater than that produced in its presence. No really satisfactory explanation of this effect has yet been put forward. At one time it was suggested that the inhibitory action of the presence of glucose on the formation of some enzymes could be attributed to the production of fermentation acids, but in many cases this has now been disproved. A further suggestion that has been put forward is that the presence of glucose during growth has a " protein sparing " action similar to that postulated in mammalian nutrition. The addition of glucose to washed suspensions of *Esch. coli* has no effect on the deaminase activities of the cells, so that glucose has no effect once the enzymes have been formed in the cell and the inhibitory action must affect enzyme formation during growth.

An alteration of the *p*H of the medium during growth does have a marked effect on enzyme production, but the suppression of deaminase formation, for instance, by the presence of glucose is greater than can be explained by the fall in *p*H due to acid formed by its fermentation. Table VI shows the action of the presence of glucose during growth on the formation of various enzymes of *Esch. coli*, and it can be seen that in some cases the effect can be satisfactorily explained by fermentation acidity, while in others the effect is greater than, or sometimes even opposed to, that produced by an equivalent acidity during growth. Only in the case of glucozymase (the enzyme system responsible for the first stage of glucose fermentation) does the presence of glucose during growth result in an enhanced activity over and above that due to acidity.

TABLE VI

COMPARISON OF THE ACTIVITIES OF *Esch. coli* WHEN GROWN IN CASEIN DIGEST: (1) adjusted to *p*H 7, (2) adjusted to *p*H 5, and (3) containing 2 per cent glucose and attaining a final *p*H = 5·2.

Activities are expressed as Q units = μl. O_2 or CO_2 or NH_3 or methylene blue (MB), etc., formed or reduced/hr./mgrm. dry weight of organism.

Enzyme	Q Unit	Activity of Organism Grown (1) at *p*H 7	(2) at *p*H 5	(3) in Glucose	Glucose Effect
Hydrogenase	MB	240	126	146	None
Catalase	O_2	4200	6360	6310	None
Arginine decarboxylase	CO_2	2	338	272	None
Lysine decarboxylase	CO_2	53	194	198	None
Alanine deaminase	NH_3	32	4	1	Inhibition
Glutamate deaminase	NH_3	12	3	1	Inhibition
Aspartase	NH_3	127	247	15	Inhibition
Serine dehydrase	NH_3	855	656	167	Inhibition
Tryptophanase	Indole	5·4	1·6	0·2	Inhibition
Alcohol dehydrogenase	MB	52	179	44	Inhibition
Succinic dehydrogenase	MB	43	23	9	Inhibition
Formic dehydrogenase	MB	110	138	58	Inhibition
Glucozymase	Glucose	38·5	31	77	Stimulation

The presence of fermentable carbohydrate during growth has four known effects: (1) the production of acid and consequent alteration of the medium *p*H; (2) the production of gas with consequent anaerobiosis; (3) a considerably increased crop of organisms; and (4) the transient formation of polysaccharide within the growing and fermenting cell. Despite various attempts, no one has yet succeeded in linking any of these effects with the inhibition of formation of certain enzymes. Monod has investigated the effect of growing the

organism in a mixture of sugars. For example, if *Esch. coli* is grown in a mixture of glucose and galactose, he finds that the organism utilises all the glucose before it begins to attack the galactose. Galactose is attacked by means of an adaptive enzyme, galactozymase, which catalyses the phosphorylation to galactose-l-phosphate, and galactozymase is not formed by the organism until all the glucose is removed from the medium. If the organism is first grown in galactose so that it contains galactozymase, and then inoculated into a mixture of glucose and galactose, the galactozymase activity disappears until all the glucose is again used up. In other words, the formation of the constitutive glucozymase suppresses the formation of the adaptive galactozymase. Monod suggests that the effect is due to a definite "enzyme suppression." There is still no clear explanation of the mechanism of this suppression, although it can be postulated that both enzymes arise from a limited supply of a common protein precursor: the formation of the constitutive glucozymase thus uses up the available precursor so that the adaptive galactozymase cannot be produced. This hypothesis involves, in turn, a further supposition that the active enzyme is produced by some reaction between substrate and precursor, and that a substrate such as glucose has a higher affinity for the precursor than a substrate such as galactose. In this connection, Spiegelman and his co-workers have shown, in yeast, that the formation of galacozymase in washed cells is accompanied by a fall in the glucozymase activity, but that if the cells are provided with available nitrogen so that they can synthesise proteins without drawing on their internal reserves, then the formation of the new enzyme can occur without reduction in other activities. All these findings emphasise that the living cell is a very dynamic system with its enzymes continually undergoing breakdown and resynthesis.

Gram-positive bacteria differ from Gram-negative organisms in that they are able to assimilate certain amino-acids and concentrate them in the free state in the internal environment. The assimilation of certain amino-acids, such as glutamic acid

and histidine, can only take place if energy is provided by some metabolic activity such as glucose fermentation. Some Gram-positive organisms also differ from many of the Gram-negative species in that they are unable to synthesise glutamic acid, etc., from ammonia, whereas many Gram-negative species can synthesise all their amino-acid requirements from ammonia. Yeasts again synthesise their amino-acids from ammonia, but cannot assimilate ammonia unless fermentation is occurring simultaneously. Further research is necessary to clarify the relation of these various findings, but there is a suggestion that the presence of glucose during growth may alter the assimilatory processes of the cells, and this, in turn, may be reflected in an alteration of the enzyme constitution, especially with regard to those enzymes concerned with the breakdown of amino-acids to ammonia.

Further examples of substances, other than the specific substrate, having an effect on enzyme formation, are found in the case of certain growth factors (see Chap. V) which act as coenzymes. For instance, *Haemophilus parainfluenzae* is unable to synthesise coenzyme I, and is unable to grow in its absence. If, however, sub-optimal amounts of coenzyme are provided in the growth medium, then we find that the organisms grow, but are unable to oxidise at a normal rate those substances forming the substrates of coenzyme I dehydrogenase systems. The deficient organisms can oxidise L-malic acid slowly, but if coenzyme is added to a suspension of these organisms they can then oxidise malic acid at the normal rate. This means that the organism has synthesised its normal complement of enzyme-protein, but has been unable to saturate it with coenzyme due to its inability to synthesise this substance.

PHYSICO-CHEMICAL CONDITIONS OF GROWTH

Aerobiosis or anaerobiosis

Facultative anaerobes can grow under strictly aerobic or strictly anaerobic conditions and develop a different enzyme constitution in each case. When we examine the activities

of individual enzymes we find that those that can function under aerobic conditions only, are produced only when growth is aerobic, being suppressed when growth is anaerobic, and vice versa. Taking the deaminases of *Esch. coli* as examples, the formation of the oxidative L-alanine deaminase is 5-6 times greater under aerobic conditions than under anaerobic, while the formation of the anaerobic serine dehydrase is 2-3 times greater when growth is anaerobic than when aerobic.

*p*H of the growth medium

Esch. coli can grow in a casein-digest medium adjusted to any *p*H between the approximate limits 4·2 and 9·5. The formation of an enzyme within the bacterial cell is dependent to a large extent upon the *p*H of the medium at the time of formation of the cell. The effect of the *p*H varies with the type and function of the enzyme concerned, and we can distinguish three types up to the present:

(*a*) NEUTRALISATION MECHANISMS: bacteria are able to grow in media covering a wide range of *p*H by the production of mechanisms whose action is to neutralise the external acidity or alkalinity and so tend to stabilise the internal environment. Thus growth in an acid medium promotes the formation of enzymes catalysing reactions with alkaline end-products, and inhibits the formation of enzymes having acid-forming actions. When *Esch. coli* grows in an acid medium containing amino-acids, it attacks certain of the amino-acids by decarboxylation liberating CO_2 with the formation of alkaline amines; when it grows in an alkaline medium the amino-acid decarboxylases are no longer formed, but, instead, enzymes attacking amino-acids by deamination are formed and these liberate NH_3 with the formation of acid products. Other organisms react to acid growth conditions by the formation of enzymes catalysing the formation of neutral substances from acids—as, for example, the reduction of butyric acid to butyl alcohol by *Cl. acetobutylicum*, and the formation of acetylmethylcarbinol from pyruvic acid by *Aerobacter aerogenes* (see Chap. VII). In all these cases

investigation of the amount of enzyme formed in the cell at various growth *p*H values shows a direct relation between the formation of the neutralising mechanism, suppression of the non-neutralising mechanisms, and the *p*H of the environment (see Fig. 2).

(*b*) PROTECTIVE MECHANISMS: the function of some enzymes such as catalase is to destroy metabolites which, if allowed to accumulate, would prove toxic to the cell. All enzymes are optimally active at a definite *p*H and, consequently, as the environment *p*H diverges from this *p*H of

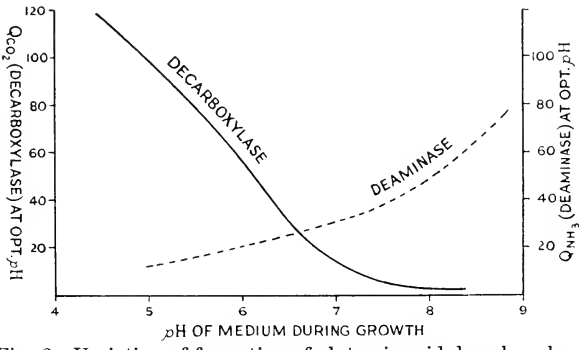

Fig. 2. Variation of formation of glutamic acid decarboxylase and deaminase of *Esch. coli* with the *p*H of the medium during growth.

optimal activity, the effectiveness of each enzyme unit decreases. This means that in the case of catalase, which has optimal activity at *p*H 6·5, the enzyme unit is considerably less effective during growth occurring at *p*H 9 than at *p*H 6·5. In such cases we sometimes find that the organism compensates for this loss of efficiency per enzyme unit by the production of more enzyme so that the effective activity (= No. of enzyme units × activity of each unit at the environmental *p*H) is roughly constant whatever the *p*H in the medium. Enzymes whose formation is affected by *p*H in this way are urease, catalase, formic, and alcohol dehydrogenases—enzymes whose

substrates are toxic to the organism. Fig. 3 shows the variation of the formation of alcohol dehydrogenase of *Esch. coli* with growth *p*H. The potential activity is that activity estimated at the optimal activity *p*H (8·0) of the enzyme and represents the total formation of enzyme within the cell, the effective activity is the activity estimated at the *p*H of the environment in which the cell was grown.

(*c*) A THIRD GROUP is formed by those enzymes whose formation is maximal when the growth *p*H approximates to the

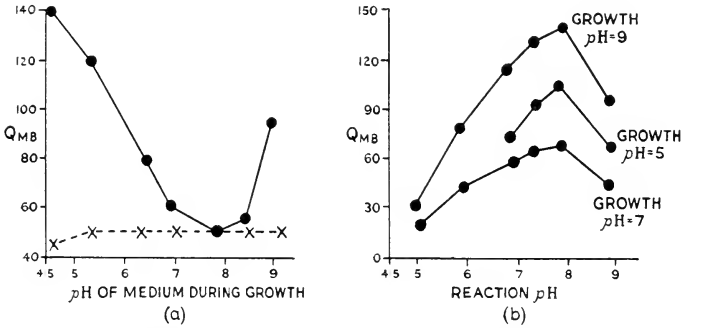

Fig. 3. (*a*) Variation of potential activity (●—●) and effective activity (x - - - x) of alcohol dehydrogenase of *Esch. coli* with *p*H of medium during growth.

(*b*) Variation with reaction *p*H of activity of alcohol dehydrogenase of *Esch. coli* grown at various *p*H values—showing that *p*H of optimum activity does not vary with growth *p*H. $Q_{MB} = \mu l.$ methylene blue reduced/hr./mg. dry weight of organism.

(After Gale and Epps, *Biochem. J.*, 1942, **36**, p. 609.)

value of their *p*H of optimum activity. It is remarkable that, as far as we know, there is no enzyme whose formation is not affected in some way or other by the environmental *p*H during growth. Enzymes of this third group, having functions neither neutralising nor protective, are formed to a significant extent over a limited range of growth *p*H values centred about the value of the optimal activity *p*H. The growth *p*H value giving maximal formation is not necessarily the same as the optimum activity *p*H, as can be seen in Fig. 4 for the case of

hydrogenase. In this example the optimum activity *p*H is 6·0 (Fig. 4*b*), but maximal formation of the enzyme occurs at *p*H 8·0 (Fig. 4*a*) and investigation of the effective activity between growth *p*H values of 6 and 8 shows that the greater formation of the enzyme between these values compensates for the loss of activity per enzyme unit over this range. As a result the effective activity is approximately constant over the middle of the growth range but falls off rapidly

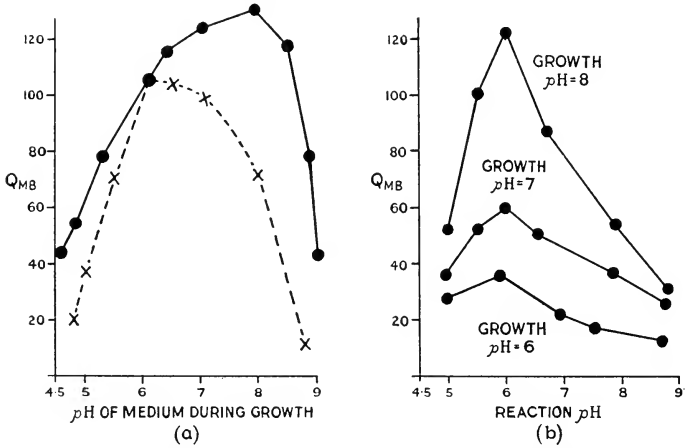

Fig. 4. (*a*) Variation of potential activity (●–●) and effective activity (x---x) of hydrogenase of *Esch. coli* with *p*H of medium during growth.

(*b*) Variation of reaction *p*H of hydrogenase activity of *Esch. coli* grown at various *p*H values. (After Gale and Epps, *Biochem. J.*, 1942, **36**, p. 612.)

outside these limits. In these cases, then, we get a restricted amount of compensatory formation over the neutral part of the growth range but no compensation towards the ends of that range. Enzymes in this group include hydrogenase, succinic dehydrogenase, glucozymase, and tryptophanase.

Growth temperature

It has become customary to study many organisms after growth at 37° C., presumably since this is the temperature of

parasitic existence in man. It does not follow that this is the optimum temperature for bacterial metabolism. In fact, many soil organisms cannot grow successfully at temperatures as high as 37° C. A few studies have been made of the effect of growth temperatures on enzyme constitution, and it has been shown, for example, that the amino-acid decarboxylases of some strains of *Esch. coli* are formed to a greater extent when growth occurs at 20° C. than when at 37° C. Several workers have shown that the efficiency of protein synthesis increases as the temperature falls.

THE AGE OF THE CULTURE

When an organism is inoculated into a suitably nutrient medium, it begins to increase in size until, in due course, the enlarged organism divides into two daughter-cells apparently similar to the mother-cell. This process will go on until some nutrient in the medium is exhausted. The growth process can be followed experimentally in two main ways: by counting the number of cells per ml. of medium, or by determining the mass of cell-material (measured as cell-nitrogen, cell-carbon, dry weight of cell-material, etc.). If we record the amount of growth against time, we find that we get curves of different shape if we measure growth by cell-numbers or by cell-mass, as shown in Fig. 5.

If we follow the increase in cell-numbers with time we find that the curve can be divided into a number of phases. Starting from the time of inoculation, we get

1. an initial stationary phase during which no increase in cell-numbers takes place;

2. a lag phase during which the rate of multiplication increases with time;

3. a phase of logarithmic growth when the rate of multiplication is constant;

4. a phase of negative growth acceleration during which the rate of growth decreases with time;

5. a maximum stationary phase.

If we count the number of viable cells (*i.e.* the cells capable of further division) rather than the total number of cells present, we find a similar curve (Fig. 5), though the number of viable cells is always less than the total number of cells present. Following the maximum stationary phase, during which the number of new cells is balanced by the number of dying cells, we get a falling off in numbers as the cells die at an increasing rate.

If, however, we estimate growth by cell-mass rather than by cell-numbers, we get a different curve, as shown in Fig. 5,

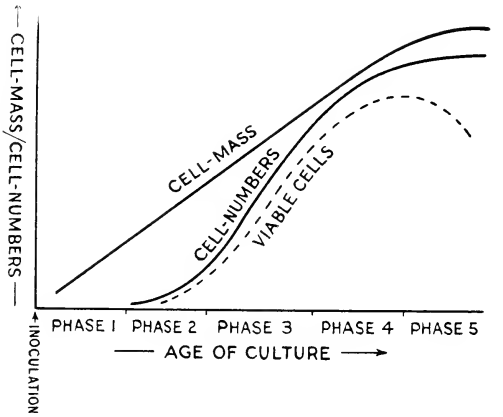

Fig. 5.

which shows no initial stationary or lag phases but a steady increase in mass until growth ceases. The difference between the two curves lies in the fact that the size of the cells is not the same throughout the growth period. When the inoculum-cells enter the new medium they begin to grow in size, *i.e.* in cell-mass, but do not divide and so give rise to the stationary phase of cell-numbers. The cells do eventually divide, but whereas they may divide at a limiting size x in the logarithmic phase of growth, they will grow to a size considerably larger than x before division occurs in the lag phase of growth. Consequently we have a steady increase in cell-mass, but

the cells themselves are, on the average, larger in phases 1 and 2 than they are in phases 3 and 4, and it is this difference in cell-size during various growth phases that gives rise to the different shapes of the growth curves. If the inoculum-cells have to undergo any form of adaptation before growth can take place in the new medium, then stationary and lag phases may be shown by the cell-mass curves as well as by the cell-number curves.

If we wish to investigate the development of an enzyme system with the growth of a culture, the results we shall obtain if we correlate enzyme activity with cell-mass will obviously differ from those we shall obtain if we correlate activity with cell-numbers. Many of the early investigations of this problem were calculated on a basis of enzyme units per cell, and curves were obtained which showed very high enzymatic activities of cells during the early phases of growth. Since the cells are larger during these phases than in the later stages of growth, it follows that they will contain more protoplasm than older cells and may well therefore contain more enzyme. We can only follow the development of the enzyme if we relate it to the amount of cell-substance present without reference to cell-numbers. Of more recent years the estimation of cell-mass has become a relatively simple matter owing to the development of photo-electric and turbidimetric methods, and nowadays the enzymatic activity of bacteria is usually expressed as enzyme units per mgrm. dry weight of organism or per mgrm. nitrogen content. Whichever form of expression is used we find marked variations of enzyme content with the age of the culture. Since the culture is formed by continued binary fission of cells, each division apparently similar to the last, it is not immediately obvious why the enzyme content of the cells should vary with the time elapsing since inoculation, but we must remember that the physico-chemical nature of the environment is changing throughout the growth period as a result of the metabolic activities of the growing cells, and it has already been shown that the enzyme content of a cell is largely dependent upon the environment at the time of its formation.

When the enzyme content of the culture is expressed on a basis of enzyme units per mgrm. dry weight of organism, we find two main types of variation with the age of the culture (Fig. 6).

In the case of enzymes giving the Type I variation (Fig. 6), cultures taken as early as possible in the growth period have high activities, and these activities decrease as the culture grows, usually falling off rapidly after cell division has ceased. In the Type II variation, cells taken early in the growth period have little or no activity and the enzyme is formed during growth, reaching a maximum at about the time of cessation

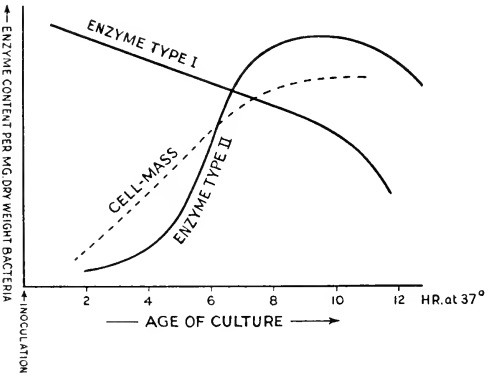

Fig. 6.

of cell division. After the end of the growth the activity may fall off, due to death of the cells, oxidation or digestion of the enzyme protein, etc. It is probable that we have not yet studied all the types of enzymes present in bacteria and the majority of those so far reported in the literature give a Type II variation with age of culture. These enzymes—deaminases, decarboxylases, dehydrogenases, etc.—are concerned with the breakdown of substrates with the liberation of carbon, nitrogen, energy, etc., and it does not follow that these enzymes have any direct connection with the synthetic processes of growth. Consequently it is possible that this Type II form of variation

with age is characteristic of enzymes concerned mainly with catabolic or protective mechanisms. There is also the possibility that some, at any rate, of these variations are artefacts produced by the permeability of the cell-wall to substrates varying with age of culture. That this is not always the case, however, has been demonstrated with extracellular enzymes such as proteases, whose formation in the external environment follows just such a curve as that shown for Type II enzymes in Fig. 6. In the case of certain amino-acid decarboxylases, showing a Type II variation, it has been possible to estimate the amount of enzyme formed in the cells by breaking these down with acetone and ether, when the amount of enzyme formed within the cell is found to vary with the age of the culture in the same way as the Type II variation found with the intact cells. If the Type II variation is characteristic of catabolic systems, it may be that Type I variation is characteristic of anabolic systems which the cell must possess for growth to take place. Our knowledge is not yet sufficiently extensive for any such generalisations to be made. In Streptococci we find enzymes showing both types of variation: tyrosine decarboxylase showing Type II; arginine dihydrolase (see p. 171) and the enzymes involved in glucose fermentation showing Type I variation.

Where the formation of enzymes within the cell is also conditioned by *p*H, the Type II variation may be modified if growth occurs in the presence of fermentable carbohydrate. Thus an enzyme whose formation is optimal only when the growth *p*H approximates to the optimum activity *p*H (*e.g.* hydrogenase) may give a Type II variation with age, but the activity may decrease again before the end of growth owing to the *p*H of the medium becoming considerably acid. These variations are very important in the experimental study of bacterial metabolism. For example, *Cl. acetobutylicum* possesses hydrogenase and acetoacetic acid decarboxylase (acetoacetic acid⟶acetone), both of which show a Type II variation with age of culture. If we study the formation of these enzymes in suspensions of organisms

harvested at different ages of culture we find that (*a*) cells harvested very early in the growth period have neither enzyme; (*b*) cells harvested during the phase of linear growth possess a very active hydrogenase but no acetoacetic decarboxylase; and (*c*) cells harvested at the time of cessation of growth have no hydrogenase activity but a very active acetoacetic decarboxylase. These differences in time of formation can probably be correlated with the fact that the *p*H of optimum activity of hydrogenase is 8·0, while that of acetoacetic acid decarboxylase is approximately 5, and the

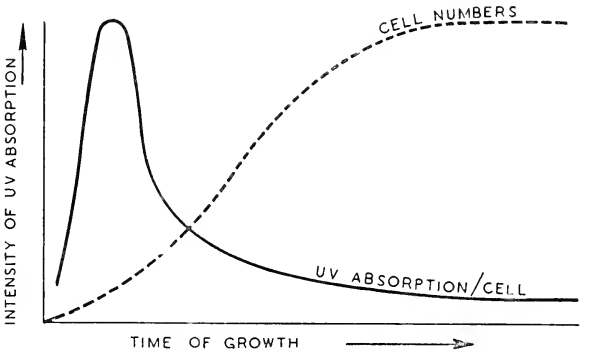

Fig. 7.

*p*H of the medium falls from 7 to *ca.* 4·5 during the growth period.

It might be thought that some changes in the chemical composition of the cell during its growth should be demonstrable in view of the marked alterations in enzymic constitution. Changes in enzymic constitution involve changes in protein constitution, but these may relate to the organisation of the amino-acids within the molecules, rather than to gross changes in their proportions. However, in recent years, workers in Sweden have applied the technique of ultra-violet spectrophotography to bacteria and have found very significant changes in the composition of the cell during growth. Substances containing purines and pyrimidines, such as nucleotides

and nucleic acids, have a very marked absorption in the ultra-violet at 265 mμ, and changes in the amount of such substances in the cells can be shown by photographing the cells in light of that wave-length. If this is done, it is found that cells from old cultures have very little "nucleic" material but that this increases markedly during the growth phases corresponding to the late lag and early logarithmic periods; the concentration then decreases steadily throughout the phases of negative growth acceleration and stationary growth. The changes in the concentration of nucleic material (*i.e.* measured by its U-V absorption at 265 mμ) are related to the growth of the organism as shown in Fig. 7. It can be seen that there may well be correlation between these alterations in composition and those variations in enzymic activity described above.

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CHAPTER V

GROWTH: SYNTHESIS OF BACTERIAL PROTOPLASM

When a bacterium is inoculated into a nutrient medium it first begins to increase in size, and this increase in cell-material is eventually followed by binary fission, the two daughter-cells proceeding to increase in size until they divide, and so on. The speed at which this process takes place depends upon the particular organism concerned and on the physico-chemical constitution of the environment. *Esch. coli* will divide once every twenty minutes when inoculated into a nutrient broth at *p*H 7 and 37° C. This means that each cell synthesises its own weight of protoplasm including proteins, enzymes, prosthetic groups, essential metabolites, etc., in twenty minutes. In this chapter we intend to survey the synthetic abilities of various organisms.

For synthesis to occur an organism requires (1) inorganic salts, (2) a source of carbon, (3) a source of nitrogen, and (4) a source of energy. Table VII summarises sources of carbon, nitrogen, and energy which are either commonly available in nutrient media or which are known to be essential for the growth of particular organisms. Some organisms can synthesise all their protoplasm from simple sources such as carbon dioxide and ammonia, plus a source of energy, and must therefore be equipped with all the enzymes necessary for the formation of the essentials of their existence from these simple sources. This is not the case with all bacteria, as many organisms are lacking in enzymes necessary for certain synthetic processes. When this occurs the organism in question is unable to synthesise some essential constituent and is consequently unable to grow unless and until that particular constituent is supplied ready made in the environment. When an organism has such a synthetic disability it is said to be nutritionally "exacting" towards the substance which it is unable to synthesise. It is by a study of the synthetic disabilities of the more exacting organisms that we gain our

knowledge of the synthetic abilities of the nutritionally non-exacting.

NUTRITIONAL REQUIREMENTS

Inorganic salts

All bacteria require the presence of certain inorganic ions for growth. Salts are required for the regulation of osmotic pressures, for the maintenance of membrane equilibria, and for the action of enzymes. Enzymes such as catalase, cytochrome oxidase, and polyphenol oxidase, possess a metal as part of their structure, phosphatases require the presence of magnesium as coenzyme, while pyrophosphatase is optimally active only in the presence of a definite ratio of magnesium and calcium ions. Traces of metals such as zinc, cobalt, molybdenum, vanadium, etc., are found to be essential for certain activities in some organisms. The presence of phosphate plays an essential role in energy transfer and many fermentation reactions. A nutrient salt mixture which will support growth must contain Na^+, K^+, Fe^{++}, Mg^{++}, Cu^{++}, NH_4^+, Cl', SO_4'', PO_4''', $CaCO_3$, and traces of other metals which are usually present in sufficient quantities as impurities in the commercial salt preparations. Recent investigations on the properties of *Esch. coli* and *Aerobact. indologenes* grown in iron-deficient media have shown that the organisms grown under such conditions are deficient in certain enzymes such as formic dehydrogenase, hydrogenase, and formic hydrogenlyase (see p. 132), with the result that they carry out an acid fermentation of glucose without the usual formation of gas (see Chap. VII). If the iron deficiency is severe, the organisms may show a decreased content of catalase and cytochrome.

Autotrophic bacteria

The least exacting group of organisms is the *autotrophic* group, members of which are able to multiply in a purely inorganic environment, synthesising their carbon substances from CO_2 or HCO_3', their nitrogenous material from ammonia or nitrate, and obtaining their energy in one of two ways.

CHEMOSYNTHETIC AUTOTROPHES: organisms of this group synthesise all their protoplasmic constituents from CO_2 or HCO_3' and NH_3 or NO_3', and obtain the energy for the synthesis by oxidation of an inorganic substrate which is specific for the particular organism, and by means of which the various organisms can be identified. For example, there are two chemosynthetic autotrophes present in soil which carry out the nitrification of ammonia. The first step in the oxidation is carried out by *Nitrosomonas*, which obtains its energy from the oxidation of ammonia to nitrite:

$$2NH_3 + 3O_2 = 2HNO_2 + 2H_2O + 79 \text{ Cals.}$$

Nitrite is then oxidised to nitrate by *Nitrobacter* utilising this reaction as a source of energy for its synthetic processes:

$$HNO_2 + O = HNO_3 + 21{\cdot}6 \text{ Cals.}$$

The oxidation substrate is specific; *Nitrobacter* cannot oxidise or grow on ammonia as source of energy, while *Nitrosomonas* cannot oxidise nitrite; neither organism can oxidise sulphite or carbon compounds. Some of the chemosynthetic organisms are inhibited by the presence of organic matter; thus the nitrifiers are inhibited by the presence of meat broth but the inhibitory substances can be removed by extraction of the broth with ether or ethanol. The growth of *Nitrobacter* is accelerated by the addition of 0·1 per cent peptone to the medium but is inhibited by the presence of asparagine, gelatine, or urea. Consequently the nitrifying organisms cannot be isolated by plating out on solid media containing gelatine or agar-agar, but are usually isolated by growth on inorganic media solidified in silicic acid gel.

A further example of a chemosynthetic autotrophe is the iron bacterium, *Leptothrix ochracea*, which lives in iron-containing streams and obtains its energy by the oxidation of ferrous carbonate to ferric hydroxide. The complete reaction is

$$4FeCO_3 + O_2 + 6H_2O = 4Fe(OH)_3 + 4CO_2 + 81 \text{ Cals.}$$

Iron bacteria can play an important part in the corrosion of iron pipes, etc.

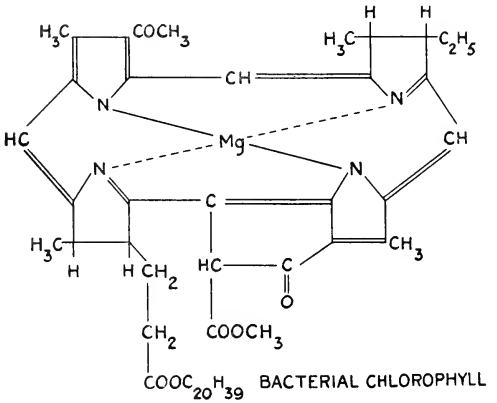
An organism of particular interest is *Thiobacillus thio-oxidans*, which utilises as energy source the oxidation of elementary sulphur to sulphuric acid:

$$2S + 3O_2 + 2H_2O = 2H_2SO_4 + 141{\cdot}8 \text{ Cals.}$$

This organism has an exceptionally high tolerance of acid, and is unaffected by a *p*H value as low as 0·6, while it grows most rapidly at a *p*H between 3 and 4. In this case the oxidation of sulphur provides the energy for the assimilation of CO_2 and its reduction to cell-carbon. The processes can be separated; the organism will oxidise a certain amount of S in the absence of CO_2 and then, if exposed to CO_2 later, will take up and reduce an amount of CO_2 corresponding to the initial oxidation. The oxidation of S is accompanied by an uptake of inorganic phosphate from the medium and this phosphate is again liberated during CO_2 reduction; this suggests that the energy obtained from the oxidation process is stored as a form of " energy-rich " phosphate until it is utilised in the reduction process. Umbreit has isolated adenosine-tri-phosphate from *Thiobacillus thio-oxidans*, which suggests that this is the organic phosphate in which the energy is stored, thus linking the energy-systems of the autotrophic bacteria with those demonstrated in heterotrophic organisms (see Chap. VII).

PHOTOSYNTHETIC AUTOTROPHES: It is obvious from the nature of their metabolism that the chemosynthetic autotrophes must be strictly aerobic in their habitat. The photosynthetic autotrophes, on the other hand, are strict anaerobes and obtain the energy for their synthetic activities by photochemical utilisation of light energy. They obtain their nitrogen from ammonia or nitrate, their carbon from bicarbonate, and reduce the bicarbonate to organic carbon by a linked oxidation of an inorganic substrate. The organic carbon so produced may be of carbohydrate nature in the first instance and can be conveniently represented by

(HCOH). Important members of this group are the sulphur bacteria *Thiorhodaceae* which are abundant in soil, mud, and sulphuretted waters. Some varieties of the group are purple and some green, the colour in each case being due to a mixture of pigments including a magnesium-porphyrin pigment with the structure of chlorophyll, differing from that of plants only in the nature of certain of the side-chains.



BACTERIAL CHLOROPHYLL

These organisms reduce CO_2 to organic carbon by a linked oxidation of hydrogen sulphide:

$$CO_2 + 2H_2S = (HCOH) + H_2O + 2S.$$

In the presence of large amounts of hydrogen sulphide, the elementary sulphur is deposited inside the cell in the case of the purple sulphur bacteria, or outside the cell in the case of the green sulphur bacteria. If the supply of hydrogen sulphide is limited, a further oxidation-reduction takes place, the elementary sulphur being oxidised to sulphuric acid:

$$2CO_2 + H_2S + 2H_2O = 2\,(HCOH) + H_2SO_4.$$

Again the autotrophic organisms are characterised by oxidation of an inorganic substrate. In the photosynthetic group the oxidation occurs anaerobically as a means of reducing

available CO_2, but in the chemosynthetic group the oxidation occurs aerobically as a source of energy.

The strict autotrophes can multiply only in the presence of inorganic matter, and may be inhibited by the presence of organic matter. There are, however, some organisms which lead an autotrophic-like existence, in that they utilise CO_2 or bicarbonate as carbon source and ammonia or nitrate as nitrogen source, but are able to obtain energy from the breakdown of certain organic substances. These organisms thus form a bridge between the true autotrophes and the heterotrophes, and are consequently called "autotrophic heterophants"; they do not represent any large proportion of the organisms in common experience.

An example of an organism whose metabolism is intermediate between that of the autotrophes and that of the heterotrophes is *B. methanicus* which obtains its energy (and possibly its carbon) by the oxidation of methane.

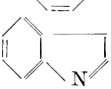
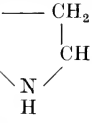
$$CH_4 + 2O_2 = CO_2 + 2H_2O + 195 \text{ Cals.}$$

We also find organisms whose metabolism is intermediate between that of the photosynthetic autotrophes and the heterotrophes. Thus the *Athiorhodaceae* are photosynthetic organisms closely related to the *Thiorhodaceae* but use organic acids as hydrogen-donators whereby to reduce the CO_2. They can be distinguished from the heterotrophes in that growth will only occur anaerobically and in the light. CO_2 is essential to the growth and the organism appears to obtain most of its carbon material from the assimilation of CO_2.

TABLE VII

COMMON SOURCES OF CARBON AND NITROGEN

Sources of Carbon	Sources of Nitrogen
CO_2	N_2
HCO_3'	NH_3
	NH_2OH
Carbohydrates:	NO_2'
Glucose	NO_3'

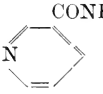
Sources of Carbon	Sources of Nitrogen
Fructose Lactose Sucrose Maltose Starch Glycogen etc.	Amino-acids (natural isomers): Glycine, $CH_2NH_2.COOH$ Alanine, $CH_3.CHNH_2.COOH$ Serine, $CH_2OH.CHNH_2.COOH$ Cysteine, $CH_2SH.CHNH_2.COOH$ Cystine, $CH_2.CHNH_2.COOH$ (S–S) $CH_2.CHNH_2.COOH$
Fatty acids: Acetic acid Propionic acid Butyric acid	Threonine, $CH_3.CHOH.CHNH_2.COOH$
Keto-acids: Pyruvic acid Acetoacetic acid Oxalacetic acid Ketoglutaric acid	Methionine, $CH_3.SCH_2.CH_2.CHNH_2.COOH$ Valine, $(CH_3)_2.CH.CHNH_2.COOH$ Leucine, $(CH_3)_2.CH.CH_2.CHNH_2.COOH$ Norleucine, $CH_3.CH_2.CH_2.CH_2.CHNH_2.COOH$ Isoleucine, $CH_3.CH_2.CH(CH_3).CHNH_2.COOH$
Hydroxy-acids: Lactic acid Malic acid	Phenylalanine,  $CH_2.CHNH_2.COOH$ Tyrosine, HO  $CH_2.CHNH_2.COOH$
Alcohols Glycerol	Tryptophan,  $CH_2.CHNH_2.COOH$
Hydrocarbons	Histidine,  $HC{=}{=}C{-}CH_2.CHNH_2.COOH$
Amino-acids	Lysine, $H_2NCH_2.(CH_2)_3.CHNH_2.COOH$ Arginine, $HN{=}C(NH_2){-}NH.CH_2.(CH_2)_2.CHNH_2.COOH$ Glutamic acid, $HOOC.CH_2.CH_2.CHNH_2.COOH$ Aspartic acid, $HOOC.CH_2.CHNH_2.COOH$ Proline, 

This table is by no means comprehensive and includes only those substances mentioned in this book.

TABLE VIII

Growth Factors

Nicotinic acid, 

Nicotinic amide, 

Coenzyme I (see p. 31)

Haematin (see p. 34)

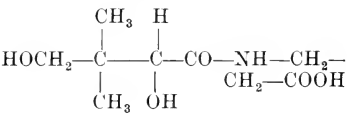
Riboflavin (see p. 33)

Thiamin (see p. 33)

β-Alanine,

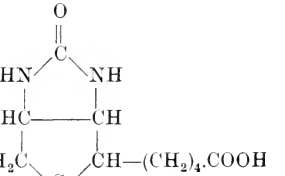
$H_2N.CH_2.CH_2.COOH$

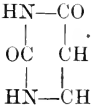
Pantothenic acid,

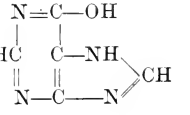


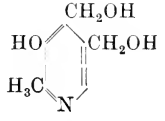
Pimelic acid,

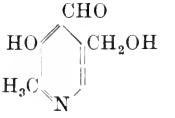
$HOOC.(CH_2)_5.COOH$

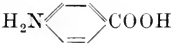
Biotin, 

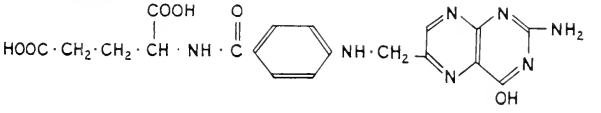
Uracil, 

Hypoxanthine, 

Pyridoxin, 

Pyridoxal, 

p-Amino-benzoic acid, 

Folic acid (*casei* factor), 

Heterotrophic bacteria

The other major group of bacteria is the *heterotrophic* group; these organisms obtain their carbon mainly from organic sources, their nitrogen from either or both inorganic and organic sources, and the energy for their synthetic processes by the degradation (oxidation, fermentation, etc.) of energy-rich organic material. Heterotrophic bacteria are thus related in their general metabolism to animals, while the autotrophic bacteria are related to plants. It is possible that the two higher forms of existence may have evolved from the related groups of micro-organisms.

Heterotrophic bacteria can be subdivided on nutritional grounds as follows (see Table IX):

(*a*) ORGANISMS ABLE TO UTILISE ATMOSPHERIC NITROGEN: The ability to trap atmospheric nitrogen and transform it into inorganic or organic nitrogenous compounds within the cell is called " nitrogen fixation," and is a property possessed by comparatively few species. Such organisms are of great importance in agriculture, since the natural fertilisation of the soil is a result of their activities. The most important member of this group is *Azotobacter*, a strict aerobe found free-living in the soil. This organism can grow in the complete absence of " fixed-nitrogen " as long as it is provided with atmospheric nitrogen and a source of carbon in the form of fermentable carbohydrate. When growth occurs under such conditions there is a quantitative relation between the amount of carbohydrate fermented and the amount of nitrogen fixed. Despite much research, we still have little or no definite knowledge of the chemistry of the fixation process, and the primary product of fixation has not yet been identified. Claims have been made that atmospheric N_2 is first reduced by the organism to either NH_3 or NH_2OH but these claims have yet to be satisfactorily substantiated. Studies in which *Azotobacter* has been exposed to gaseous nitrogen, enriched with isotopic-N_2, have shown that the isotope appears in the amino-acids of the organism within a few minutes of exposure. The

TABLE IX

CLASSIFICATION OF BACTERIA ACCORDING TO THEIR NUTRITIONAL REQUIREMENTS

	Source of Carbon		Source of Nitrogen			Growth Factors	Source of Energy		
	CO_2 HCO_3'	Organic C	N_2	NH_3 NO_3'	Organic N		Light	Inorg. Oxidtn.	Organic Decomp.
1. Autotrophes:									
(*a*) chemosynthetic	+	−	−	+	−	−	−	+	−
(*b*) photosynthetic	+	−	−	+	−	±	+	−	−
2. Autotrophic: heterophants	+	−	−	+	−	−	−	−	+
3. Heterotrophes:									
(*a*) N-fixers	(+)	+	+	(+)	−	−	−	−	+
(*b*) non-exacting	(+)	+	−	+	−	−	−	−	+
(*c*) amino-acid exacting	(+)	+	−	+	+	−	−	−	+
(*d*) growth factor exacting ...	(+)	+	−	+	−	+	−	−	+
(*e*) exacting towards both amino-acids and growth factors ...	(+)	+	−	+	+	+	−	−	+

EXAMPLES:

1 (*a*). *Nitrosomonas; Nitrobacter.* 1 (*b*). *Thiorhodaceae.* 2. *B. methanicus.* 3 (*a*). *Azotobacter.*
3 (*b*). *Esch. coli.* 3 (*c*). *Eberthella typhosa.* 3 (*d*). *Pr. vulgaris.* 3 (*e*). *S. haemolyticus.*

various amino-acids have been isolated and their content of the isotopic-N determined; those containing the highest amount of isotope must be those which are formed first, as a result of the fixation process. Glutamic acid is found to contain the highest content of isotopic-N and so, presumably, is the first amino-acid formed after fixation and must act as precursor of the other amino-acids. The organisms will grow on ammonia as N-source but if such fixed-nitrogen is provided in the medium, then fixation immediately ceases. If the organism is supplied with ammonia enriched with isotopic-N, it can utilise this immediately and the isotope is found in the cell-protein within three minutes of contact. The distribution of the isotope in the amino-acids of the cell is the same as that obtained when the cell is fixing isotopic-N_2; this result suggests that ammonia is a primary product of the fixation reaction. When growth of *Azotobacter* is occurring by fixation of atmospheric nitrogen then the presence of traces of iron, calcium, and molybdenum are essential. The greater requirement of molybdenum for growth on gaseous nitrogen than for growth on ammonia-nitrogen suggests that this metal has a function in the fixation process.

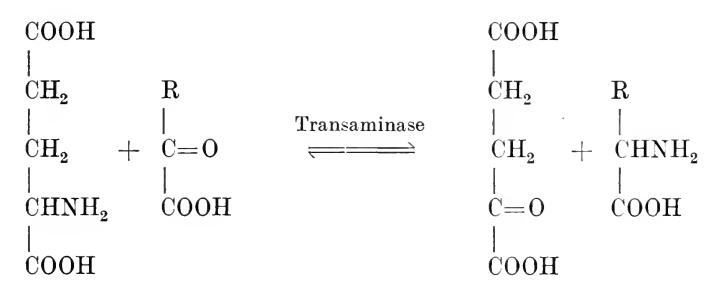
Nitrogen fixation is not confined to *Azotobacter*, but is also a property of some photosynthetic bacteria and certain *Clostridia* such as *Cl. pastorianum*, which was the first nitrogen-fixing organism to be isolated from soil. In addition, the *Rhizobaceae*, the root-nodule bacteria, can perform nitrogen-fixation, but only when living in symbiosis with the host-plant (see Chap. X).

(*b*) NON-EXACTING ORGANISMS: This is probably the largest of the sub-groups and consists of those organisms which can synthesise their nitrogen requirements from ammonia or nitrate, their carbon from a simple organic source such as glucose or lactate, and obtain their energy from the degradation of organic matter. *Esch. coli* is a typical example, in that it can grow luxuriantly in a medium consisting of nutrient salts, including ammonium ions, and either glucose or lactate as

carbon and energy source. The organism can be maintained indefinitely by serial subculture in such a medium.

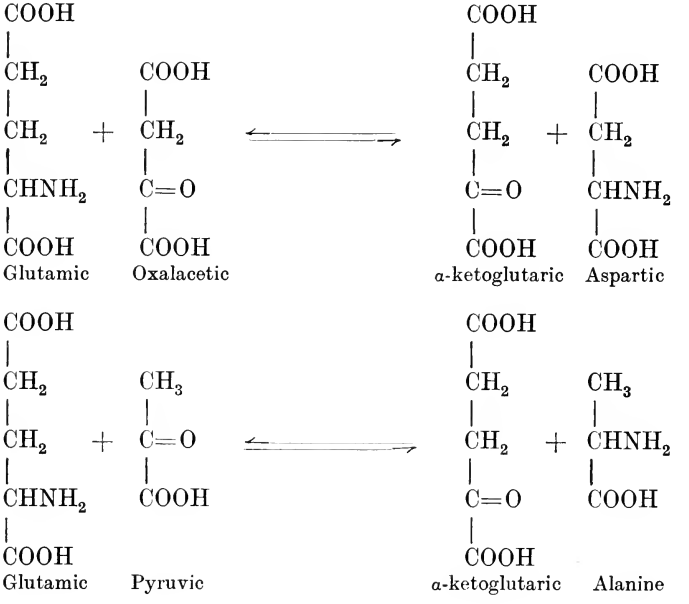
(*c*) ORGANISMS EXACTING TOWARDS CERTAIN AMINO-ACIDS: The bacteria belonging to the sub-groups so far discussed are able to synthesise all their amino-acids from a source of inorganic nitrogen and a suitable source of carbon. Analysis of the proteins of bacteria shows that they resemble all other proteins in being composed of some twenty-odd amino-acids, all of which can be synthesised by these organisms from ammonia and a source of carbon such as glucose.

Aspartic acid can be synthesised from fumaric acid and ammonia by the enzyme aspartase (see p. 162), while glutamic acid can be synthesised from α-ketoglutaric acid and ammonia by reversal of the glutamic dehydrogenase system (p. 47). In mammalian tissues glutamic acid, and to a smaller extent aspartic acid, act as the starting point for the formation of other amino-acids by a process of transamination whereby the amino-group of the dicarboxylic amino-acid is transferred to the α-position of an α-keto-acid:



and the α-ketoglutaric acid so formed is then resynthesised to glutamic acid through the reversal of the glutamic dehydrogenase system. This reaction was originally discovered by Braunstein and Kritzmann, and it has been suggested that any α-keto-acid can enter into the reaction, so that transamination opens up a way for the general synthesis of other amino-acids from glutamic acid and the corresponding keto-acids. Since

the original work, other investigators have obtained cell-free preparations of transaminating enzymes, and these appear to catalyse, *in vitro*, transamination between glutamic acid and oxalacetic acid or between glutamic acid and pyruvic acid, but not to carry out a general transamination as first suggested.



The only "new" amino-acid formed in this way is alanine from pyruvic acid. The prosthetic group of transaminase is pyridoxal phosphate and if an organism has lost the ability to synthesise pyridoxin (see p. 33) then it cannot produce active transaminase. Recent work with Lactobacilli, which have lost the ability to synthesise pyridoxin, has shown that the organisms can grow in a medium which contains D-alanine even in the absence of pyridoxin. Organisms grown in this medium are devoid of pyridoxin and its derivatives, whereas organisms grown in media containing pyridoxin but no

D-alanine are found to synthesise D-alanine. D-alanine cannot be the precursor of pyridoxin but it is probable that pyridoxin mediates the synthesis of the unnatural isomer of alanine. This finding has led to the discovery of a new enzyme called "racemase" which produces DL-alanine from L-alanine. D-alanine is essential for growth and is synthesised by transaminase followed by racemase, both enzymes having pyridoxal phosphate as prosthetic group. It appears improbable that transaminase is concerned with the general synthesis of amino-acids, as first suggested, but it is always possible that the cell-free preparations and the studies carried out with them represent only a part of a more complex system within the living cell.

The transamination reactions:

Glutamic acid + oxalacetic acid → α-ketoglutaric acid + aspartic acid,

Glutamic acid + pyruvic acid → α-ketoglutaric acid + alanine,

have now been demonstrated for many bacteria including species of *Escherichia*, *Shigella*, *Eberthella*, *Proteus*, *Pseudomonas*, *Azotobacter*, *Staphylococcus*, *Streptococcus*, and *Pneumococcus*. A cell-free transaminase has been prepared from *S. faecalis* and resolved into specific protein and a prosthetic group replaceable by pyridoxal phosphate.

The biosynthesis of other amino-acids has been elucidated by the application of a new technique which is peculiar to microbiology. It has been mentioned above (see p. 60) that the formation of enzymes in the cell is determined by the presence of the controlling gene and that alteration of that gene results in the loss of that enzyme. The rate of alteration or mutation of genes can be accelerated by irradiation with X-rays and this method has been used by Tatum, Bonner, Beadle, and their co-workers to produce a very large number of artificial mutants of the bread mould *Neurospora crassa*. The same method was later applied to other moulds, such as species of *Penicillium* and *Aspergillus*, and to bacteria such as

Escherichia coli, but the early work which involved genetical analysis of the mutants produced was carried out with *Neurospora*. The synthesis of cellular material occurs as the end-result of a chain of reactions, each catalysed by a specific enzyme. If an essential cell constituent D is synthesised from the raw food-stuff A by a series of steps $A \rightarrow B \rightarrow C \rightarrow D$, then the cell will be able to grow if it is supplied with any of the substances A, B, C, or D. If, however, the enzyme catalysing the formation of C from B is inactivated, then the organism will be able to grow provided it is supplied with either D or C but not if supplied with A or B. If we can find three mutants of the organism, each of which has lost one of the enzymes involved in the synthesis of D from A, then, by studying the nature of substances necessary for growth of the mutants, it should be possible to reconstruct the chain $A \rightarrow B \rightarrow C \rightarrow D$. This is the principle of the method of "biochemical mutants."

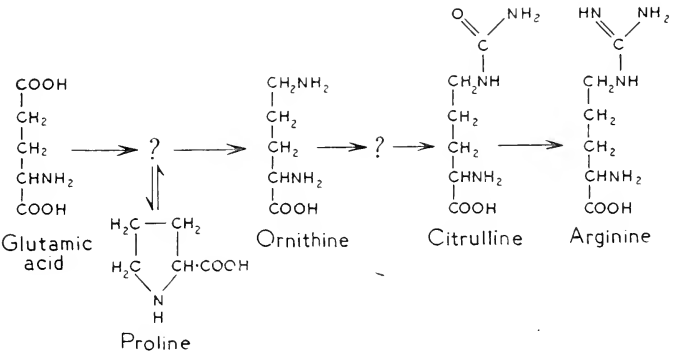
Irradiation of *Neurospora* results in the production of mutants; the absorption of one quantum of radiant energy causes an alteration of one gene which causes the loss, in turn, of one enzyme. Some of the mutants produced will have lost an enzyme involved in the synthesis of an amino-acid X. All the "wild type" organisms can grow on a medium which contains ammonium ions as N-source but the mutants will only grow if the amino-acid X is added to the medium. Consequently the organisms obtained after irradiation are examined for their ability to grow (1) on a medium containing ammonia as sole N-source, and (2) on a medium containing ammonia + X as N-source. All those organisms which grow on (1) can be discarded, while those which grow only on (2) are further investigated. In the first place the number of genetic types present is determined; this gives an idea of the number of genes, and consequently enzymes, involved in the synthesis of X. Next the possible precursors of X are tested as nutrients in place of X.

For example: seven different mutants of *Neurospora* were isolated by Srb and Horowitz and were found to require

arginine for growth; of the seven, one would grow on addition of nothing but arginine, two would grow on citrulline or arginine, and four would grow on ornithine, citrulline, or arginine. This indicates that the biosynthesis of arginine from some precursor N must take place according to the sequence:

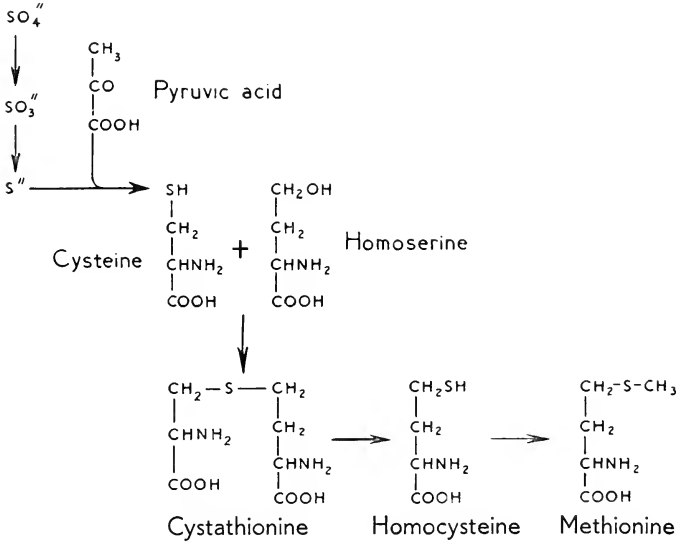
$$N \rightarrow (\text{four separate steps}) \rightarrow \text{Ornithine} \rightarrow ? \rightarrow \text{Citrulline} \rightarrow \text{Arginine}.$$

The same synthetic series was investigated by Bonner, using mutants of *Penicillium*, and he was able to elucidate some of the earlier stages of the sequence and to show that glutamic acid is a precursor of arginine thus:

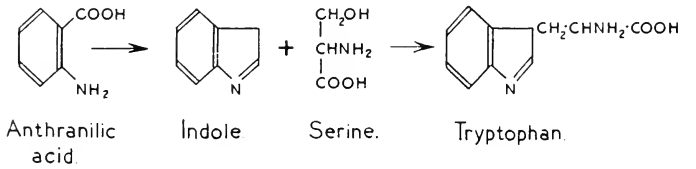


Thousands of such biochemical mutants of various micro-organisms have now been isolated and the nutritional requirements of a small fraction of them discovered. Many of these show a disability in the synthesis of an amino-acid and their detailed investigation is yielding much information on the biosynthetic precursors of substances such as valine, isoleucine, methionine, tryptophan, lysine, etc. It is not known yet whether results obtained with one organism also apply to another, although it is fairly certain that the biosynthesis of tryptophan is very similar in *Neurospora* and *Escherichia coli*. The synthesis of methionine from sulphate has been worked

out with *Esch. coli* and the last four steps confirmed in *Neurospora*:



Biochemical mutant studies with *Neurospora* have shown that the biosynthetic precursors of tryptophan are anthranilic acid and indole, and that the last step involves the condensation of indole with serine. This condensation has been further proved by the preparation of a cell-free enzyme which accomplished the synthesis in the presence of pyridoxal phosphate as prosthetic group:



These syntheses have been deduced from studies with artificially-induced biochemical mutants but mutation also occurs spontaneously and, consequently, mutants of bacteria, etc., will arise which have lost the ability to synthesise amino-acids. This is the case with freshly isolated strains of *Eberthella typhosa* which are unable to synthesise tryptophan and consequently cannot grow in a tryptophan-free medium. If a trace of tryptophan is added to the basal medium of salts, ammonia and glucose, then normal growth and subculture is possible. The organisms are able to grow if provided with indole so the enzyme which has been lost catalyses a step in the biosynthesis of indole rather than of tryptophan itself. Fildes has shown that it is possible to select tryptophan-synthesising mutants from the non-synthesising culture and so, presumably, obtain the primitive synthetically competent strain (see p. 63). *Eberthella typhosa* is exacting, if at all, to tryptophan alone, but other species, especially Gram-positive cocci, are unable to synthesise other amino-acids and consequently will grow only in a medium rich in preformed amino-acids.

If a cell has lost the ability to synthesise its amino-acids, it necessarily becomes dependent upon the supply of these substances in the medium. The concentration and relative proportions of the various amino-acids in the medium will rarely be those optimal for protein synthesis by the organism, and it has been shown recently that certain Gram-positive bacteria, such as the Streptococci and Staphylococci, have acquired a concentration mechanism which compensates for this loss of synthetic ability. These organisms possess a cell-wall or membrane which enables them to take up amino-acids from the external environment and to concentrate them inside the cell prior to metabolism or condensation into protein. Basic amino-acids such as lysine are able to diffuse through this cell-wall, but acidic amino-acids such as glutamic acid cannot penetrate the wall unless energy is supplied to the cell through a metabolic process such as fermentation. If the cell ferments glucose, then glutamic acid passes rapidly through the

cell-wall and becomes concentrated within the cell to an extent such that the internal concentration may be over a hundred times that in the medium. Gram-negative organisms do not possess this capacity to concentrate amino-acids in the free state inside the cell.

(*d*) ORGANISMS EXACTING TOWARDS GROWTH FACTORS: Some species of bacteria are able to grow in complex media such as blood-serum, yeast extract, etc., but are unable to do so in a salt-ammonium-glucose medium, even if a mixture of pure amino-acids is added. In such cases, fractionation of the blood or yeast medium leads to the isolation of a substance or substances whose presence in minute quantities is essential to growth, and which is known as a "bacterial vitamin" or "growth factor." Table VIII gives a list of some of the bacterial growth factors that have been identified. They have no common chemical nature. A simple example is given by the organism *Pr. vulgaris*, which is unable to grow in a salt-ammonia-glucose medium unless nicotinic acid or nicotinic amide is added. The presence of 1×10^{-8} grm. nicotinic acid per ml. medium is sufficient to support full growth. From Table VIII it can be seen that several of the growth factors are either prosthetic groups or parts of prosthetic groups of proteins and enzymes, and it would seem that some bacteria find difficulty in synthesising these chemically complex active groups of enzyme systems. *Pr. vulgaris* is unable to synthesise nicotinic acid and consequently cannot manufacture coenzymes I and II, essential for the action of certain oxidation mechanisms. In the case of *Haemophilus parainfluenzae*, the synthetic disability extends to the nicotinamide-nucleoside part of the coenzyme molecule, and growth cannot take place in the presence of nicotinic acid or amide, but only if the nucleoside or the complete coenzyme molecule is added to the medium. A sub-maximal growth of this organism can occur in the presence of sub-optimal amounts of coenzyme in the medium, and such "deficient" organisms have an impaired oxidation mechanism, in that the rate of oxidation of certain substrates is considerably less than normal.

If washed suspensions of these deficient organisms are prepared and their rate of oxidation of malic acid studied (malic acid dehydrogenase requires coenzyme I), it is found that the addition of coenzyme I to the suspension during the test results in a greatly increased rate of oxidation. This demonstrates that the organism has synthesised the enzyme malic dehydrogenase, but the activity of the enzyme is not fully effective as the coenzyme part of the system is deficient, and the addition of coenzyme repairs the deficiency.

In this last example it was possible to grow an exacting organism in the presence of sub-optimal quantities of a growth factor whose function could be guessed with reasonable certainty, and then demonstrate a metabolic impairment in that function. A similar technique can be used to determine the metabolic function of other growth factors; to do this a culture is grown in the presence of sub-optimal quantities of growth factor and a control culture in the presence of excess growth factor. A survey is then made of the metabolic activities of the two cultures in an attempt to discover an activity affected by the deficiency of growth factor in the deficient culture. If such an impaired activity is found, the effect on the activity of adding growth factor to the washed suspension of the deficient organism is studied. *Staph. aureus*, for example, is exacting towards thiamin. Thiamin-deficient organisms metabolise pyruvic acid at a rate significantly less than that of normal organisms, and the addition of thiamindiphosphate makes good the deficiency. It follows that thiamin plays some part in the metabolism of pyruvic acid by these organisms.

Studies on the impairment of metabolism in growth-factor-deficient cultures have assisted in the elucidation of the function of pyridoxal which is the biologically active form of pyridoxin (see Table VIII). If we study the growth of streptococci in media containing increasing amounts of pyridoxin, but otherwise fully nutrient, we get a curve relating the amount of growth to the pyridoxin content of the medium as shown in Fig. 8. We can distinguish three types of culture:

cells grown in medium A, rich in pyridoxin; cells grown in medium B, containing just sufficient pyridoxin to allow full growth; and cells grown in medium C, which is deficient in pyridoxin to such an extent that the growth is seriously restricted by the pyridoxin deficiency. If we investigate the activity of the two enzymes, tyrosine decarboxylase (p. 168) and transaminase (pp. 93-4), in the three cultures, we find that both enzymes are fully developed in culture A; in culture B the transaminase system is fully developed, but the

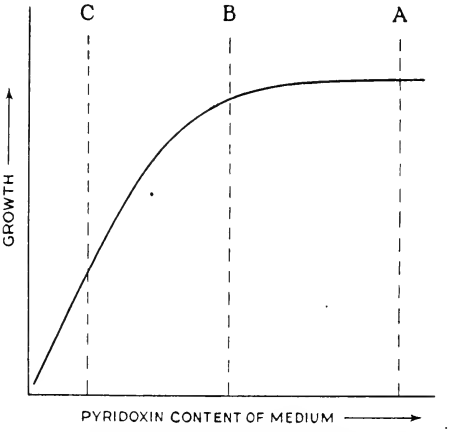


Fig. 8. Relation of growth of streptococci to pyridoxin content of growth medium.

decarboxylase has about 5 per cent of the activity of culture A; in culture C the transaminase activity is considerably less than that of cultures A and B, and no decarboxylase activity is demonstrable. If, however, we take the cells from cultures B and C and re-estimate their activities in the presence of added pyridoxal, we find that both the tyrosine decarboxylase and transaminase activities are restored to normal. This suggests that the organism has synthesised the protein portions of the enzymes in all three cultures, but that the enzymes are inactive in the absence of their prosthetic groups or coenzyme

moieties which are related to pyridoxin in structure. Detailed studies of the cell-free enzymes have now proved that both enzymes have the same prosthetic group, and this can be substituted *in vitro* by pyridoxal phosphate. The actual identity of the natural prosthetic group with pyridoxal phosphate has yet to be established, although there is little doubt but that they are the same.

Some at least of the growth factors thus seem to function as prosthetic groups of essential enzyme systems. The synthetic disability may refer to the whole prosthetic group, as in the case of riboflavin for *S. haemolyticus*, or to a part of the essential molecule as in the cases of nicotinic acid for *Pr. vulgaris* and of thiamin for *Staph. aureus*. It is highly probable that other growth factors are also needed by the organism as parts of essential prosthetic groups. The nature of the essential metabolism involved can be shown by the deficient culture technique, and then, later, studies of the cell-free enzymes can establish the complete structure of the prosthetic group containing the growth factor. This type of investigation is now being used to elucidate the structure of the active forms of the growth factors pantothenic acid and biotin. Pantothenic acid-deficient cultures of organisms, which require this substance as growth factor, are found to have impaired metabolism of acetic acid and impaired ability to carry out acetylation reactions. Thus deficient cultures of *L. plantarum* have a greatly impaired ability to acetylate choline and this ability can be restored by the addition of pantothenic acid to the washed cells. Lipmann (see p. 34) has isolated a sulphanilamide acetylase from animal tissues and shown that it possesses a new prosthetic group, which he calls " coenzyme A," which contains pantothenic acid. Concentrates of coenzyme A have now been shown to act as the prosthetic group of several enzyme preparations from bacteria and yeast which catalyse reactions involving acetate or acetyl phosphate. Thus a preparation which catalyses the condensation of acetyl phosphate and oxalacetate to yield citrate is activated by coenzyme A. Coenzyme A has not yet been obtained in a

pure state but there is little doubt that it is a compound containing pantothenic acid as part of its structure. The position of biotin is very similar: deficient culture studies have shown that biotin-deficiency results in impairment of oxalacetate formation from pyruvic acid and CO_2, and in decreased activity of aspartic acid and serine deaminases (see p. 163). Cell-free preparations of aspartic acid deaminase can be activated by biotin and adenylic acid; both factors are necessary and it is fairly certain that the preparations contain a second enzyme which brings about the synthesis of a co-aspartase from them. A combined form of biotin has been isolated from yeast extracts and this material will act as co-aspartase. Its structure has not yet been determined.

In most of these cases we find that the biologically active structure (= prosthetic group) is a more complex molecule than the growth factor. This can be explained by saying that the synthesis of the growth factor is more difficult for the organism to accomplish than that of the rest of the prosthetic group but, on the mutational explanation of variation, it is probably more correct to say that mutations involving loss of synthetic ability towards the growth factor give the organism a greater energetic advantage (or " selection pressure ") in a complex medium than those involving loss of ability to synthesise the rest of the molecule. Whatever the explanation, it does not follow that all strains of an organism which is nutritionally exacting towards, say, pantothenic acid will display a disability towards the whole molecule. Some organisms can synthesise pantothenic acid if they are supplied with β-alanine. Others, which apparently require biotin, can synthesise this factor if supplied with the pimelic acid part thereof. In these cases, the lost enzymes are concerned with the synthesis of the β-alanine portion of pantothenic acid or the pimelic acid portion of biotin respectively.

Many growth factors have been discovered by analysis of complex " satisfactory " media, but one, *p*-amino-benzoic acid, was first discovered as an antagonist to the drug sulphanilamide (see p. 113) and then shown to be a growth factor for, first,

Cl. acetobutylicum. Its metabolic function is still not clear. It has been shown to form part of the structure of folic acid, a growth factor for several Lactobacteriaceae, but it may have functions other than those involved in the production of folic acid. Folic acid itself is presumably a prosthetic group but no enzymes requiring it have yet been discovered. The deficient-culture technique does not seem to have yielded results, but the discovery that folic acid can be replaced in growth media by mixtures of thymine and other purines and pyrimidines has led to the suggestion that the folic-activated enzymes are concerned with purine and pyrimidine synthesis. Similar evidence suggests that it may also be concerned in methionine synthesis while *p*-amino-benzoic acid, apart from folic acid, may be involved in the synthesis of other amino-acids, such as lysine.

A growth factor of unknown constitution and function is Strepogenin. This is a substance necessary for the growth of some Lactobacteriaceae and has the properties of a peptide. It can be obtained from partial hydrolysates or enzymatic digests of crystalline proteins such as insulin and so must represent a part of the structure of these proteins. It is thought that it is a specific peptide structure synthesised by a specific enzyme which can be lost by mutation.

Haemophilus influenzae is exacting towards haematin, but can in some cases dispense with this if growth takes place under anaerobic conditions. It would appear that haematin is necessary for some oxidative process, and since catalase is a haematin-enzyme, it has been suggested that the haematin is required for the synthesis of catalase which protects the organism from hydrogen peroxide formed during aerobic existence. This suggestion is supported by the fact that haematin can be replaced as a growth factor by cysteine, which decomposes H_2O_2 by reduction.

In the same way that it is possible to select mutants of *Eberthella typhosa* which can synthesise their own tryptophan, it is also possible in some cases to select from cultures of organisms exacting towards growth factors, mutants which

will be able to synthesise these factors. Thus it is possible to "train" *Staph. aureus* to synthesise thiamine by serial sub-cultivation in media containing progressively less of this factor.

(*e*) ORGANISMS EXACTING TOWARDS BOTH AMINO-ACIDS AND GROWTH FACTORS: It has been suggested that an organism becomes exacting when it grows for many generations in a medium in which all growth requirements are provided ready made. If the rate of growth is regulated by the rate of synthesis of some factor, and that factor is provided ready made in the environment, then the organism will be able to grow more rapidly if it utilises the preformed factor than if it is dependent upon the synthetic process. Likewise, a mutant which has lost the ability to synthesise that factor will grow as well as, if not better than, the synthetically competent cell in the rich medium. Rich media would therefore be expected to select nutritionally-exacting mutants and, in general, the richer the medium, the poorer would become the synthetic abilities of the organisms using it as a natural habitat. Soil organisms can find few or no complex growth factors in their habitat, but organisms that have assumed a parasitic existence in animal tissues are living in an environment rich in all those substances that go to make up protoplasm. In general, we find that the more parasitic an organism, the more exacting are its growth requirements. Some organisms such as *S. haemolyticus* are exacting towards several amino-acids and several growth factors. The omission of any one of these amino-acids or factors from the medium renders it sterile towards this organism. The organism is highly parasitic because only in the presence of tissues and tissue products will such an array of factors be found naturally. Parasitism leads to exacting growth requirements, and the exacting nature of the growth requirements makes parasitism obligatory. It does not follow that exacting organisms are all parasitic on man, or that they are pathogenic, as pathogenicity depends upon factors other than parasitism (see Chap. XI). Organisms such as the *Lactobacilli* from milk or

Cl. acetobutylicum from molasses, are highly exacting as a result of constant growth in these complex but inanimate media.

Table X outlines the growth requirements of various selected species that are mentioned in this book.

Carbon dioxide requirement

A distinction has been drawn between the autotrophic and heterotrophic bacteria partly on the grounds that the former utilise carbon dioxide as sole source of carbon, while the latter utilise organic material as carbon source. It is not true, however, that the heterotrophic bacteria are unable to utilise carbon dioxide, for carbon dioxide is actually essential for the growth of many, if not all, heterotrophic species. Whereas the autotrophes utilise carbon dioxide as sole source of carbon, the heterotrophes require traces only as a source of certain essential carbon compounds. The requirement of carbon dioxide was demonstrated by Gladstone and others, who showed that if simple media are rendered CO_2-free, then many heterotrophic organisms cannot grow or their growth is greatly delayed, *e.g.* neither *Esch. coli* nor *Eberthella typhosa* will grow in the absence of carbon dioxide, while the growth of *Staph. aureus* is greatly delayed, presumably until the metabolic activities of the inoculum have produced a threshold concentration. The full function of carbon dioxide has not yet been elucidated, but it has been shown in the case of the fermentation of many organisms such as *Esch. coli*, the *Propionibacteria*, etc., that carbon dioxide assimilation is involved in the formation of succinic and other 4-carbon dicarboxylic acids (see Chap. VII).

KNOWLEDGE OF SYNTHETIC PROCESSES FROM GROWTH REQUIREMENTS

By studying the growth of bacteria in mixtures of pure chemicals, we are able to divide organisms up into nutritional groups along the lines indicated above. We start, on the one

TABLE X

GROWTH REQUIREMENTS OF VARIOUS BACTERIA

	Salts	Ammonia	Co_2	Organic Carbon	Tryptophan	Specific Amino-acids	Nicotinic acid	Coenzyme I	Pantothenic acid	Pyridoxin	Thiamin	Uracil	Riboflavin	Biotin	Haematin	Pimelic acid	*p*-Amino-benzoic acid	Unknown factors
Nitrosomonas	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Azotobacter	+			+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Esch. coli	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Eberthella typhosum	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Proteus vulgaris	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Proteus morganii	+	+	+	+	−	+	+	−	+	−	−	−	−	−	−	−	−	+
H. parainfluenzae	+	+	+	+	+	+	−	+	−	−	−	−	−	−	+	−	−	−
Cl. acetobutylicum	+	+	+	+	−	−	−	−	−	−	−	−	−	+	−	+	−	−
Corynebact. diphtheriae ...	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−
Cl. sporogenes	+	+		+	+	+	−	−	−	−	−	+	−	−	−	−	−	+
Staph. aureus	+	+	+	+	+	+	+	−	−	−	+	+	+	−	−	−	−	−
S. haemolyticus	+	+	+	+	+	+	−	−	+	+	−	−	+	−	−	−	−	−
Lactob. casei	+	+	+	+	+	+	+	−	+	−	−	−	+	−	−	−	−	+

hand, with a chemically defined medium, and from that we grow a certain amount of bacterial protoplasm; in most cases we have no knowledge of the intermediate chemistry and metabolism. Exacting nutritional requirements show us what certain organisms cannot do, and we make the assumption that these synthetic disabilities of some organisms are synthetic abilities in others. To what extent is this assumption justified?

In the first place, we are able to trace a parallelism between exactingness and parasitic existence which suggests that the former arises from constant growth in the presence of complex essential substances of difficult synthesis; in some cases we are able to demonstrate a recovery of synthetic ability, such as that towards tryptophan of exacting *Eber. typhosa*, serially subcultivated in media containing progressively less tryptophan, or that towards thiamin of *Staph. aureus* subcultivated similarly in media containing progressively less thiamin.

Secondly, in the case of certain growth factors such as nicotinic acid, thiamin, pantothenic acid, pyridoxal, etc., we are able to show a metabolic impairment of deficient cells which is not found in non-exacting organisms, and in such cases the metabolic impairment is related to those enzymes which, as we know from studies in other tissues, have a structure involving the growth factor as prosthetic group or coenzyme.

Thirdly, by surveying the parts of a complex growth factor which are required by various exacting organisms, we can often show that some organisms can synthesise certain portions of the molecule but not others, *e.g.* some strains of the diphtheria organism require β-alanine as growth factor, while others require pantothenic acid; pantothenic acid will replace β-alanine for the former organisms, but β-alanine cannot replace pantothenic for the latter; consequently the former organisms cannot synthesise β-alanine, but, given that, can synthesise pantothenic acid, whereas the latter strains have a wider disability in that they cannot accomplish this further step but require the complete pantothenic acid molecule.

Fourthly, we can demonstrate the presence of the growth factors in the constitution of non-exacting organisms by the technique of microbiological assay (see below). For example, if we make up a salt-ammonia-glucose medium and carefully free it from nicotinic acid before inoculating with *Pr. vulgaris* then no growth will occur. Consequently we can use the appearance of growth as a test for the presence of nicotinic acid and, over a certain range, the growth is proportional to the amount of nicotinic acid added. In this way we can show the presence of nicotinic acid in the protoplasm of non-exacting bacteria by boiling the cells and adding the sterile extract to the basal medium. The test is highly specific and, in an analogous manner, we can show the presence of all these growth substances in the protoplasm of non-exacting organisms (*e.g.* autotrophes).

So we get knowledge concerning the synthetic abilities of nutritionally non-exacting organisms from studies of the disabilities of exacting types. Autotrophic bacteria must be able to synthesise all their amino-acids from ammonia and carbon dioxide; the amino-acids are then condensed in various stereochemical combinations to form proteins; some of these proteins require the synthesis of complex prosthetic groups before becoming active as enzymes; some enzymes are not complete without carrier systems of coenzyme I nature; the enzyme systems break down energy-yielding substances in the environment with the production of acid, alkaline, and toxic end-products, and further enzymes are synthesised to neutralise or detoxicate such products. At each stage of synthesis some organism finds the task too difficult, so we get differentiation into autotrophic, heterotrophic, anaerobic, exacting, parasitic, pathogenic, etc., organisms, and in each case the differentiating property is a reflection of the synthetic abilities of the organisms concerned.

Microbiological assay

In this chapter organisms have been mentioned which require the presence of certain growth factors in the medium before

growth can occur. Many, if not all, of these growth factors are of importance in mammalian nutrition, but are difficult to estimate by chemical means owing to their complex structure and the very small amounts in which they occur naturally. If an organism is exacting towards a growth factor F, and is inoculated under standardised conditions into a series of media containing amounts of F varying from nil to sufficient to give complete growth, then the growth is found to vary with the amount of F in the medium as shown in Fig. 9.

Over a certain range of concentrations of F there is a linear, or approximately linear, relation between the concentration

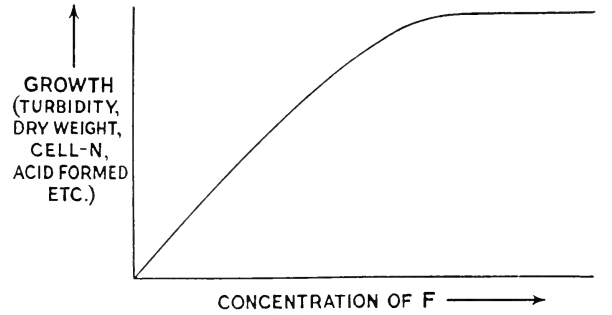


Fig. 9.

and the amount of growth. If this relation can be standardised consistently, then the growth can be used as a measure of the amount of F in a given solution. This method of growth factor or vitamin determination is known as microbiological assay. For example, the growth of *Pr. vulgaris* can be used for the assay of nicotinic acid. The method is open to many sources of error: the basal medium must be completely and easily freed from the factor to be assayed; the growth curve must be accurately reproducible; and the growth must not be affected by any other variable factor in the growth medium or in the preparation of material added for assay. The method can often be made to work with reasonable accuracy

for solutions of the assay factor, but difficulties arise when attempts are made to assay the factor in biological materials, food-stuffs, etc., as, unless it is possible to extract the factor easily and quantitatively, addition of the preparation containing the factor is certain to involve the addition of salts, amino-acids, and other growth factors which may affect the growth and so vitiate the assay. The problem is to obtain a satisfactory basal medium such that the addition of the assay factor alone has any effect on growth. Growth may be estimated turbidimetrically, but in the case of the *homo*lactic fermenters such as Lactobacilli or *S. lactis*, it is possible to obtain a measure of the growth by titration of the acid formed in the medium. Many workers prefer the titration method to the turbidity measurement. Microbiological assay is used for the estimation of nicotinic acid, pantothenic acid, biotin, pyridoxin, riboflavin, folic acid, thiamin, and certain amino-acids.

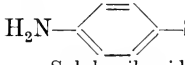
NUTRITIONAL ANTAGONISM

We have seen that many pathogenic organisms have become nutritionally exacting as a consequence of parasitic existence. Such organisms are unable to grow in media which do not contain certain growth factors, or in fully nutrient media if the utilisation of the growth factors is prevented. The possibility of preventing growth by interference with growth factor utilisation was brought into prominence by the work of Woods on sulphanilamide action.

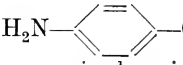
Sulphanilamide, or *p*-amino-benzene-sulphonamide, in low concentrations prevents the growth of certain bacteria, particularly the Gram-positive cocci. It can be shown that sulphanilamide does not immediately kill bacteria in these low concentrations, but prevents their division. Organisms whose growth has been checked by sulphanilamide can proceed to grow and multiply normally after removal of the sulphanilamide. This can be demonstrated by inoculating a sensitive organism into medium containing just sufficient sulphanilamide to prevent its growth; if the static culture is

now subcultured into fresh sulphanilamide-free medium, growth will occur as usual. Sulphanilamide is consequently said to be "bacteriostatic," in that it prevents multiplication without necessarily killing the organisms. The quantity of sulphanilamide which is bacteriostatic for any given organism varies greatly with the constitution of the medium in which the test is carried out. For instance, media which contain peptone or yeast extract can support growth in the presence of much higher concentrations of sulphanilamide than simple synthetic media. This is explained in the case of yeast extract by the presence in the extract of a substance which is antagonistic to sulphanilamide in that it prevents its bacteriostatic action on the bacteria. Woods investigated the properties of this anti-sulphanilamide substance, and showed that it has the properties both of a weak organic acid and of a diazotisable aromatic amine. He then tested the anti-sulphanilamide activity of *p*-amino-benzoic acid, and found that 1 molecule can neutralise the bacteriostatic action of 5000-25,000 molecules of sulphanilamide. No other substance of this nature that was tested has such marked anti-sulphanilamide activity and, shortly after, other workers were able to isolate *p*-amino-benzoic acid itself from yeast extracts. In the extracts *p*-amino-benzoic acid exists in the free state and also combined as a peptide with glutamic acid, and the amount of (free + combined) substance is sufficient to account for the total anti-sulphanilamide activity shown by the extract. At the time of the demonstration of its anti-sulphanilamide action, no function had been attributed to *p*-amino-benzoic acid in bacteria, but within a few months two Australian workers, Rubbo and Gillespie, were able to show that it acts as a growth factor for *Cl. acetobutylicum*. The list of organisms which require *p*-amino-benzoic acid as a growth factor has now been extended to include certain *Acetobacter*, *Lactobacilli*, and a mutant strain of *Neurospora*. Recently *p*-amino-benzoic acid has been shown to form part of the molecule of folic acid (Table VIII) which is required by many of the *Lactobacteriaceae*.

Fildes and Woods proposed that *p*-amino-benzoic acid is an " essential metabolite " for bacteria, and that if its metabolism is in any way prevented, then growth ceases. Since sulphanilamide and *p*-amino-benzoic acid have similar chemical structures, it was suggested that sulphanilamide acts as a competitive inhibitor of the enzyme carrying out the essential metabolism of *p*-amino-benzoic acid.



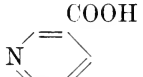
Sulphanilamide



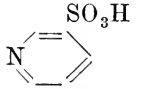
p-amino-benzoic acid

The fact that folic acid contains *p*-amino-benzoic acid as part of its structure, indicates that the latter substance must undergo some metabolism within the organism in order to become incorporated in the larger molecule.

According to this theory, *p*-amino-benzoic acid is an " essential metabolite," and if the organism has no power to synthesise this metabolite, then it becomes a growth factor for that organism. Sulphanilamide acts by preventing the utilisation of *p*-amino-benzoic acid. If this is the case, then it should be possible to inhibit the growth of other exacting organisms by presenting them with substances of structure similar to that of their specific growth factors (" metabolite analogues ") which will compete with the growth factor for an enzyme surface, will block the metabolism of the growth factor, and, consequently, prevent growth. For example, *Pr. vulgaris* is exacting towards nicotinic acid; if we add pyridine-3-sulphonic acid to the medium, we find that growth is prevented by competition between the growth factor, nicotinic acid, and its antagonistic analogue, pyridine-3-sulphonic acid.



Nicotinic acid



Pyridine-3-sulphonic acid

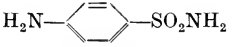
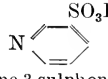
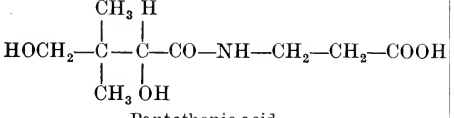
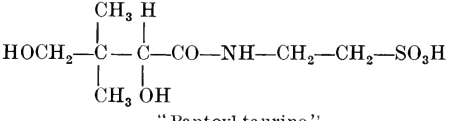
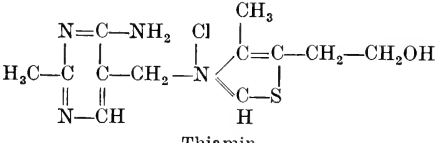
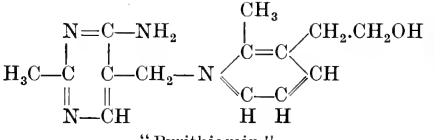
In this case it is interesting that pyridine-3-sulphonic acid acts as an antagonist towards nicotinic acid, but not towards nicotinic amide, so presumably the antagonist prevents the

synthesis of the amide from the acid. Many examples of this growth factor antagonism have now been worked out and some are given in Table XI. There is one difference between these examples and that of sulphanilamide, in that whereas the latter is effective against many organisms whether these are nutritionally exacting towards *p*-amino-benzoic acid or not, the nutritional antagonists are effective as growth inhibitors only in those cases where the organism tested is nutritionally exacting towards the factor concerned. Whether this is a difference of principle or degree remains to be seen.

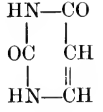
The main interest of this type of work was that it gave promise of the rational development of chemotherapeutic agents. Many nutritionally exacting organisms are pathogenic, and if it were possible to prevent their growth *in vivo* by nutritional antagonism, then the growth factor analogues might form a valuable source of chemotherapeutic agents. Pantoyl-taurine is effective as a bacteriostatic agent against streptococcal infections in the rat and is antagonised by pantothenic acid. So far, however, no marked advances in the chemotherapeutic field have come from this research for three main reasons: most of the antagonists so far prepared are simple molecules, and are excreted too rapidly to be effective *in vivo*; the internal environment of the host contains such quantities of the natural growth factor that the competitive amounts of analogue that must be injected are unreasonably large; and in some cases the effect of the analogue is to deprive the host of the metabolism associated with the growth factor as well as the organism (*e.g.* the administration of pyrithiamin to rats gives rise to the symptoms of vitamin B_1 deficiency).

TABLE XI

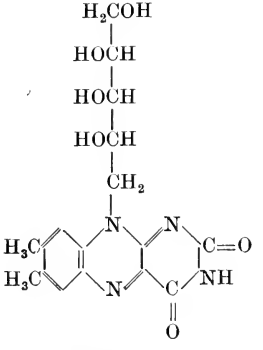
Nutritional Antagonists

Growth Factor	Antagonist	Susceptible Organisms
 *p*-amino-benzoic acid	 Sulphanilamide	*Cl. acetobutylicum* and many others
 Nicotinic acid	 Pyridine-3-sulphonic acid	*Pr. vulgaris*
$R.CHNH_2.COOH$ Certain amino-acids	$R.CHNH_2.SO_3H$ Sulphonic acid analogues	*Staph. aureus*
 Pantothenic acid	 "Pantoyl-taurine"	*S. haemolyticus* *S. lactis* *L. arabinosus* *Propionibacteria*
 Thiamin	 "Pyrithiamin"	*Staph. aureus*

$NH_2.CH_2.CH_2.COOH$
β-alanine

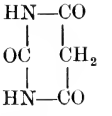


Uracil

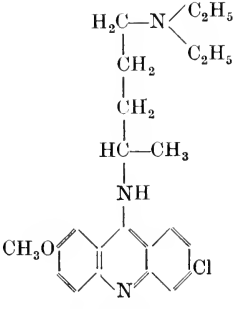


Riboflavin

$NH_2.CH_2.CH(CH_3).COOH$
α-methyl-β-amino-propionic acid



Barbituric acid



Mepacrine

Yeast

Staph. aureus

S. haemolyticus

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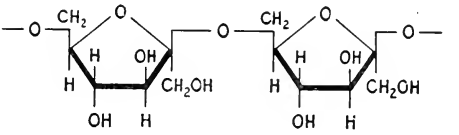
CHAPTER VI

BACTERIAL POLYSACCHARIDES

The polysaccharides synthesised by bacteria have received detailed attention from carbohydrate chemists, as not only do they present a wide range of new structures, but the polysaccharide found in the capsules of some organisms determines their immunological specificity. Polysaccharides are formed by bacteria in capsules, extracellular gums and slimes, and probably as stores of energy.

Gums and slimes

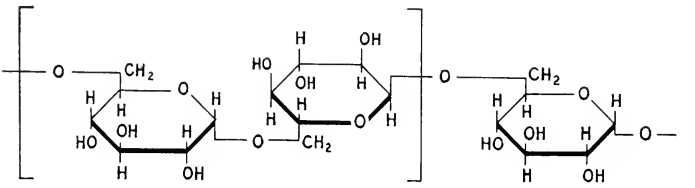
One of the earliest studies of Pasteur was concerned with the " viscous fermentation " occurring in sugar solutions, and he showed that the slime formation was due to infection of the material with certain organisms. We know now of several species of bacteria which are able to synthesise hydrophilic polysaccharide gels which are composed mainly of either glucosan (" dextran ") or fructosan (" levan "). Organisms such as *B. mesentericus* and *B. subtilis*, when growing in the presence of sucrose, give rise to a fructosan in which the fructofuranose residues are linked as follows:—



The organisms attack sucrose to form fructosan, but cannot produce the gum from glucose or fructose alone, a mixture of glucose and fructose, or from invert sugar. This suggests that energy is required to link the fructofuranose residues, and this energy is derived from the hydrolysis of the sucrose molecule. A cell-free enzyme has been obtained from *B. subtilis* which will carry out the synthesis of fructosan from sucrose:

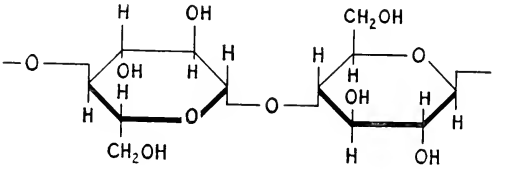
$$\text{sucrose} \longrightarrow \text{fructosan} + \text{glucose}.$$

In other cases the extracellular gum or slime is a glucosan. Organisms such as *Leuconostoc dextranicus*, *Betacoccus arabinosus*, etc., will synthesise glucosan when grown in the presence of sucrose. The glucosan has a long chain structure in which the repeating unit is



Bacterial cellulose

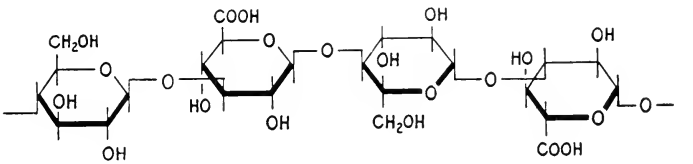
Acetobacter xylinum (see p. 149) produces a slimy envelope when growth takes place in the presence of sucrose or glycerol. The envelope is composed of a polysaccharide which has the same structure as that of vegetable cellulose, *i.e.* a chain structure consisting of cellobiose as the repeating unit:



Capsule polysaccharides

Many organisms produce capsules and these are often, though not invariably, composed largely of polysaccharides. These are of considerable interest since they appear to confer specificity upon the immunological response. For example, the genus *Pneumococcus* can be divided into 32 types by serological methods. If a serum to Type I is prepared by injection of the intact organism into an animal, then that serum reacts with Type I *pneumococcus* only. However, if a serum is prepared against the proteins of the Type I *pneumococcus*—in the absence of the polysaccharide—then the

serum will react with any pneumococcus irrespective of type. Further, the serum prepared against the intact cell will precipitate the Type I polysaccharide, but not polysaccharides prepared from other types, while the serum prepared against the protein fraction will not precipitate any of the polysaccharides. It seems, then, that the antigen is the protein part of the cell, but the presence of the polysaccharide confers specificity upon the antibody response. This suggests that the 32 pneumococcal types differ in the nature of the polysaccharides composing the capsules. The structures of several of these substances have been worked out; the Type III polysaccharide consists of glucose and glucuronic acid residues linked into chain formation as follows:—



Energy stores

In mammalian tissues, energy is stored in the glycogen deposits while in plant tissues storage occurs mainly in the form of starch. Bacteria are known to accumulate reserves of polysaccharide material within the cell, but few studies have been made as yet on the nature of these stores. For example, when *Esch. coli* is allowed to metabolise glucose in excess, then polysaccharide formation occurs within the cell, but this material is itself metabolised as soon as the external glucose is exhausted.

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CHAPTER VII

PROVISION OF ENERGY: FERMENTATION

In the last chapter we considered the materials which are essential for the growth of various bacterial species. The growth process involves assimilation of these materials and their elaboration into the constituents of the living cell. These cellular constituents are often far more complex than the nutrient materials; for example, the autotrophic bacteria synthesise protein molecules from ammonia and carbon dioxide. In other words, the energy content of the cell constituents is higher than that of the raw materials and, consequently, energy has to be supplied before cell synthesis and growth can occur. The gain in energy of the cell constituents is obtained by degradation of other energy-rich materials in the environment. The bacterial cell often obtains its energy by the degradation of carbohydrates in the environment, and this degradation can be accomplished anaerobically, in which case the process is called " fermentation," or aerobically by oxidation processes. In this chapter we shall consider the fermentation process.

The products of bacterial fermentation are many and varied, and it has been shown in Chap. II that bacteria can often be separated and differentiated on the basis of their fermentation reactions considered with respect to the sugars fermented and the products formed from those sugars. It is undesirable to deal here with the whole range of bacterial fermentations and, indeed, many have not yet been worked out in detail. In some cases bacterial fermentation provides an easily controlled method for the production of a commercially valuable substance such as butyl alcohol, and in others the production of an easily identified and specific product can be used as a characterisation test. In these cases detailed investigations have been carried out with the intention of elucidating the metabolism involved. The problem is also of interest to the biochemist interested in carbohydrate metabolism, and the fermentation of *Esch. coli* and *Aerobacter aerogenes* has received

such detailed attention that our knowledge of the intermediate stages is now almost as great as that of the related glycolysis cycles in yeast and other cells. The key substance in many fermentations is pyruvic acid, $CH_3.CO.COOH$, which is formed by the breakdown of the carbohydrate molecule and is then attacked in various ways by different organisms to give a variety of products. In this chapter we shall first trace the course of the formation of pyruvic acid and then show how various organisms produce their varied fermentation products by further elaboration of this key substance.

FERMENTATION OF GLUCOSE

Glucose is the carbohydrate whose fermentation has been studied in greatest detail. Some organisms, including many of the Streptococci and Lactobacilli, carry out a simple fermentation of glucose with the production of lactic acid in almost theoretical yield; such organisms are called *homo*-lactic fermenters. The majority of heterotrophic organisms produce a variety of products amongst which can be listed the following:—

CO_2	Pyruvic acid, $CH_3.CO.COOH$	Acetone, $CH_3.CO.CH_3$
H_2	Butyric acid, $CH_3.CH_2.CH_2.COOH$	iso-Propyl alcohol, $CH_3.CHOH.CH_3$
Formic acid, $H.COOH$	Ethyl alcohol, $CH_3.CH_2OH$	Succinic acid, $HOOC.CH_2.CH_2.COOH$
Acetic acid, $CH_3.COOH$	*n*-Propyl alcohol, $CH_3.CH_2.CH_2OH$	Acetylmethylcarbinol, $CH_3.CO.CHOH.CH_3$
Propionic acid, $CH_3.CH_2.COOH$	Butyl alcohol, $CH_3.CH_2.CH_2.CH_2OH$	2.3.Butylene glycol, $CH_3.CHOH.CHOH.CH_3$
Lactic acid, $CH_3.CHOH.COOH$		Diacetyl, $CH_3.CO.CO.CH_3$

Fermentation releases energy. From this point of view it is a less efficient form of metabolism than oxidation, for the complete oxidation of glucose yields considerably more energy than any fermentation can do, as the latter involves the production of partially reduced substances:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + 674 \text{ Cals.}$$

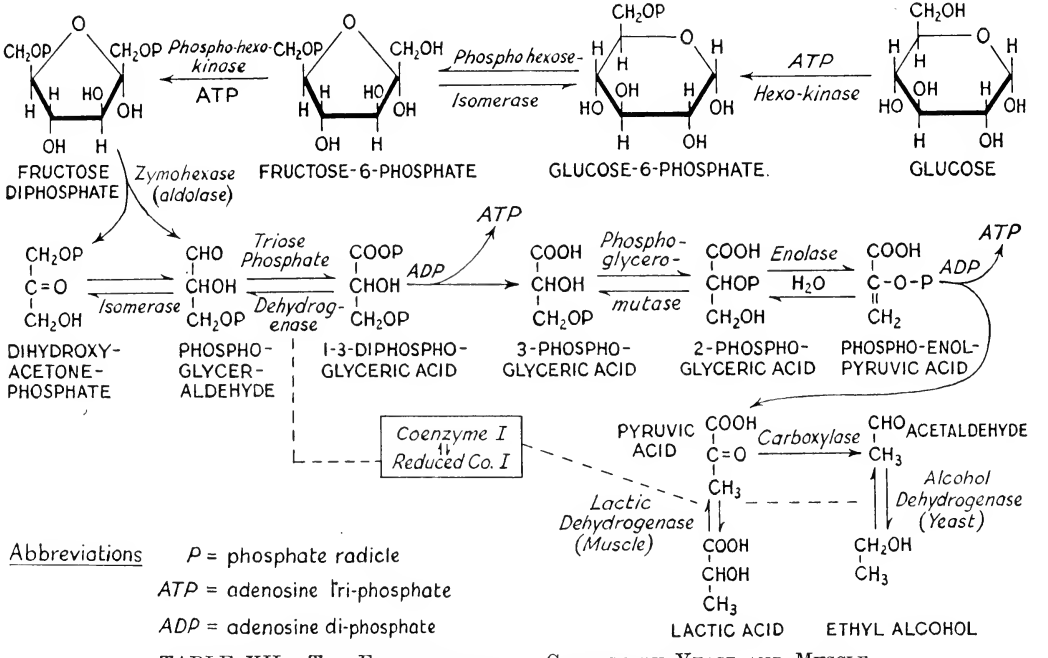
$$C_6H_{12}O_6 \longrightarrow 2CO_2 + 2C_2H_5OH + 22 \text{ Cals.}$$

A further consequence of obtaining energy by fermentation is the accumulation of large quantities of waste products which may be toxic to the organism in high concentration. Consequently this form of metabolism is necessarily restricted to small organisms living in a liquid medium, in which the waste products are quickly removed by diffusion from the immediate environment.

The anaerobic breakdown of glucose has been studied in considerable detail of recent years, and great advances have been made in our knowledge of the processes that take place during this breakdown ("glycolysis") in muscle and yeast cells. In the case of these cells it is comparatively easy to make cell-free extracts of the cells and, from these, to make preparations of the various enzymes present. In this way it has been possible to disentangle the various steps in the series of reactions and to isolate the enzymes catalysing these steps. Our knowledge of bacterial glycolysis has lagged behind that for yeast and muscle cells, as it is only of recent years that efficient methods have been discovered whereby bacterial cells can be disrupted and their enzymes liberated in an active state. Consequently there are still gaps in our knowledge of bacterial fermentation processes, and much of the work has been concerned so far with investigating whether the stages of breakdown of glucose by bacteria are the same as those occurring in yeast. As far as the processes concerned in the formation of pyruvic acid are concerned, the answer appears to be that these processes are essentially the same in bacteria as in yeasts and in various other tissues that have been investigated.

BREAKDOWN OF GLUCOSE BY YEAST

Table XII outlines the steps and enzymes involved in the breakdown of glucose to pyruvic acid by yeast cells. The first step consists of a phosphorylation of glucose to glucose-6-phosphate by the enzyme hexokinase, which catalyses the transfer of the phosphate group from adenosine-tri-phosphate



Abbreviations

P = phosphate radicle

ATP = adenosine tri-phosphate

ADP = adenosine di-phosphate

TABLE XII. The Fermentation of Glucose by Yeast and Muscle.

(ATP) to the 6-position in the glucose molecule. Glucose-6-phosphate is then altered to fructose-6-phosphate by the action of the enzyme phosphohexose-isomerase (sometimes called oxoisomerase) and a second phosphate enters the molecule in the 1-position, the second phosphate again being transferred from ATP, but under the action of the enzyme phosphohexokinase in this case. This series of reactions results in the formation of hexosediphosphate from glucose, two molecules of phosphate being taken up from two molecules of ATP with the formation of adenosine-di-phosphate in each case.

Hexosediphosphate then splits into an equilibrium mixture of triosephosphates under the action of the enzyme zymohexase (also called aldolase). The two triosephosphates are glyceraldehyde-phosphate and dihydroxyacetone-phosphate, and their interconversion is catalysed by the enzyme isomerase. We are mainly concerned with the breakdown of glyceraldehyde-phosphate in fermentation reactions and, as this is removed, dihydroxyacetone-phosphate isomerises to form more glyceraldehyde-phosphate, so that eventually the whole of the hexosediphosphate that is broken down by zymohexase can pass through the series of reactions starting with glyceraldehyde-phosphate.

Glyceraldehyde-phosphate is oxidised by the enzyme triosephosphate dehydrogenase (or glyceraldehyde-phosphate dehydrogenase). This enzyme catalyses the transfer of hydrogen from its substrate to coenzyme I, and is only active in the presence of inorganic phosphate. The immediate product of the oxidation is 1.3.diphosphoglyceric acid:

$$\begin{array}{l} \mathrm{CHO} \\ | \\ \mathrm{CHOH} + \mathrm{H_2O} + \text{coenzyme I} + \text{phosphate} \\ | \\ \mathrm{CH_2OPO_3H_2} \end{array} \xrightarrow[\text{dehydrogenase}]{\text{Triosephosphate}} \begin{array}{l} \mathrm{COOPO_3H_2} \\ | \\ \mathrm{CHOH} + \text{reduced} \\ | \qquad\qquad \text{coenzyme I} \\ \mathrm{CH_2OPO_3H_2} \end{array}$$

and the reduced coenzyme I is available as H-donator for other reactions. The diphosphoglyceric acid can give up the second phosphate to either adenylic acid or adenosine-diphosphate resynthesising ATP in the presence of the necessary

enzyme. Consequently this particular step in the breakdown process has three results:

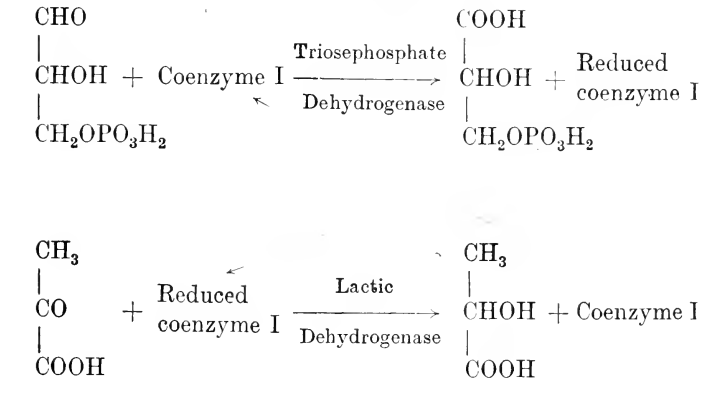
1. The oxidation of glyceraldehyde-phosphate to phosphoglyceric acid.

2. The formation of reduced coenzyme I as H-donator.

3. The uptake of inorganic phosphate and its synthesis into ATP.

Phosphoglyceromutase now catalyses the transfer of the phosphate group from the 3-position in glyceric acid to the 2-position, and water is removed from 2-phosphoglyceric acid under the action of enolase. Phospho-enol-pyruvic acid is produced which can lose its phosphate by transfer to adenylic acid or adenosine-di-phosphate with the formation of pyruvic acid and the regeneration of ATP. The phosphorylation of glucose to hexosediphosphate involves the dephosphorylation of two molecules of ATP, while the further breakdown of each molecule of glyceraldehyde-phosphate regenerates two molecules of ATP.

In muscle, pyruvic acid is reduced to lactic acid by lactic dehydrogenase working in reverse and utilising the reduced coenzyme I as H-donator. The oxidation of glyceraldehyde-phosphate and the reduction of pyruvic acid are thus linked by coenzyme I acting as H-carrier between the two enzyme systems:



In yeast cells pyruvic acid is decarboxylated by the enzyme carboxylase, and the acetaldehyde so formed is reduced to ethyl alcohol by alcohol dehydrogenase acting in reverse in a manner analogous to the lactic dehydrogenase of muscle,

$$CH_3.CO.COOH \xrightarrow{\text{Carboxylase}} CH_3CHO + CO_2$$

$$CH_3.CHO + \underset{\text{coenzyme I}}{\text{Reduced}} \xrightarrow[\text{Dehydrogenase}]{\text{Alcohol}} CH_3.CH_2OH + \underset{}{\text{Coenzyme I}}$$

THE FORMATION OF PYRUVIC ACID FROM GLUCOSE BY BACTERIA

In the scheme described above for the breakdown of glucose by yeast, the initial stages consist of a phosphorylation of the glucose molecule to form hexosediphosphate. The majority of bacteria are unable to ferment glucose in the absence of phosphate. This can be demonstrated very easily in some cases by centrifuging the organisms out of culture, washing them very thoroughly, and then incubating the washed suspension of organisms with glucose in the presence and absence of phosphate. If the washing has been successful in removing phosphate from the organisms, then fermentation will often not occur in the absence of phosphate, though it proceeds normally in its presence. This constitutes *a priori* evidence that phosphate is involved in the fermentation processes, but the existence of the enzymes catalysing the intermediate steps involved in the conversion of glucose to hexosediphosphate has yet to be proved in bacteria. It is highly probable that the same reactions occur, as *Esch. coli*, for example, will ferment fructosediphosphate to the same fermentation products as those obtained from glucose. Also when glucose is being fermented by the cells, there is an uptake of inorganic phosphate from the medium, and if sodium fluoride (which inhibits enolase) is added to the fermentation mixture, phosphoglyceric acid can be isolated as the chief

product. This suggests that the same cycle of reactions is occurring in *Esch. coli* as previously demonstrated in yeast.

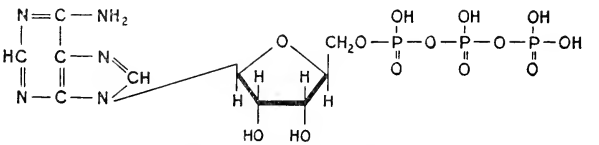
If we assume that the initial steps in the breakdown are those shown in Table XII, then the rest of the chain of reactions has been proved. Using various methods for the disruption of cells (see Chap. III), it has been possible to obtain cell-free preparations of zymohexase (aldolase), isomerase, glyceraldehyde-phosphate dehydrogenase, phosphoglyceromutase, enolase, lactic dehydrogenase, coenzyme I, and adenosine-tri-phosphate from *Esch. coli*, *Aerobacter aerogenes*, etc. There is little doubt then but that the main glycolysis cycle, as set out in Table XII, can occur in these bacteria. In general, the enzymes that have been isolated from bacteria are essentially similar in properties to their counterparts in other cells.

Liberation of energy

The formation of pyruvic acid from glucose via the cycle described above, results in the liberation of energy which is available to the organism for growth purposes. This energy is made available by the building up and subsequent rupture of the various phosphate bonds in the cycle. The energy content of phosphate bonds differs with the nature of the bond. Thus phosphate ester bonds of the type we get in hexose-diphosphate (—$CH_2OPO_3H_2$) liberate comparatively little energy when they are broken, but enol-phosphate bonds (—$COPO_3H_2$) of the type we find in phospho-enol-pyruvic acid are energy-rich, their rupture releasing about 12,000 cals. per gram-molecule compared with 3,000 cals. liberated by the rupture of an ester-bond. Glucose is thus phosphorylated at a low energy level to hexosediphosphate, and this is built up by the cycle of reactions to give the energy-rich bond in phospho-enol-pyruvic acid. Energy cannot, of course, be created and phosphoglyceric acid and phospho-enol-pyruvic acid have approximately the same energy content, but whereas the energy of phosphoglyceric acid is distributed over the

whole molecule, in phospho-enol-pyruvic acid the energy is concentrated in the phosphate bond.

A second energy-rich phosphate bond is built up during the oxidation of glyceraldehyde-phosphate with the uptake of inorganic phosphate to yield 1.3.diphosphoglyceric acid. In the presence of ADP, the diphosphoglyceric acid yields 3.phosphoglyceric acid and ATP. If the complete cycle is now inspected from the point of view of phosphate bond formation (Fig. 10) it can be seen that the initial phosphorylation of glucose to hexosediphosphate involves the utilisation of two molecules of ATP, and that the hexosediphosphate then splits to yield two molecules of triosephosphate *each* of which is eventually converted to pyruvic acid, liberating two molecules of ATP in the course of the metabolism. Thus two molecules of ATP are required to start a cycle which yields four molecules of ATP, a net gain of two molecules of ATP. The pyrophosphate bond (—P—O—P—) of ATP is energy-rich and this appears to be the form in which the cell stores its energy until it is required. The synthetic mechanisms of the cell require energy which is obtained by reactions involving phosphorylated intermediates obtained, in turn, by interaction with the extra ATP formed by the glycolysis cycle. The cycle can thus be looked upon as a machine for taking in glucose and phosphate at a low energy level, winding the energy up into specific bonds, and then transferring that energy, in the form of such bonds, to a suitable store while discarding the waste product as, in this case, fermentation products.



Adenosine-tri-phosphate

Bacteria differ from other tissues mainly in the way in which they dispose of the pyruvic acid thrown out as a waste product from the energy machine and so, in considering the further

details of bacterial fermentation, we are concerned largely with the methods utilised by the various organisms for the disposal of their waste material—although, in some cases, the organisms dispose of this material in such a way that more energy is made available during the disposal process.

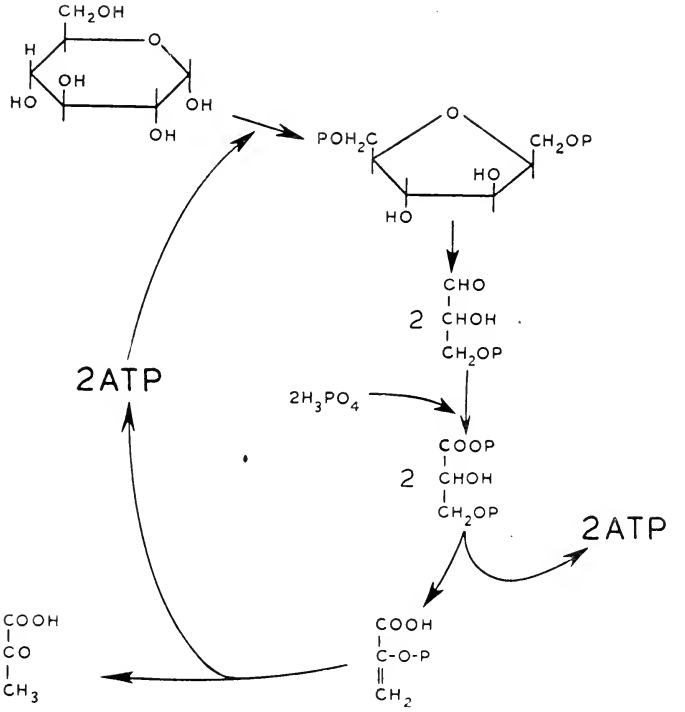


Fig. 10.

THE FERMENTATION OF PYRUVIC ACID BY *ESCH. COLI*

Acetic and formic acid formation

Under aerobic conditions, *Esch. coli* oxidises pyruvic acid directly to acetic acid.

$$CH_3.CO.COOH + O \longrightarrow CH_3.COOH + CO_2.$$

Under anaerobic conditions the first breakdown of pyruvic acid is to acetic and formic acids by what was originally called the " hydroclastic split ":

$$CH_3.CO.COOH + H_2O \longrightarrow CH_3.COOH + H.COOH \quad \ldots\ldots(1)$$

However, it was found that when cell-free enzyme preparations were used, this reaction would not take place unless phosphate was present. This suggests that the formation of acetyl phosphate occurs as an intermediate step in the breakdown,

$$CH_3.CO.COOH + H_3PO_4 \rightleftharpoons CH_3.COOPO_3H_2 + H.COOH \quad \ldots\ldots (2)$$

If the enzyme preparation is freed from adenylic acid or adenosine-di-phosphate (ADP), then acetyl phosphate is actually found to accumulate, but in the presence of either adenylic acid or ADP, the acetyl phosphate breaks down to acetic acid and the phosphate group is transferred to the adenine compound with the synthesis of ATP:

$$CH_3.COOPO_3H_2 + ADP \longrightarrow CH_3.COOH + ATP \quad \ldots(3)$$

In the intact organism the over-all result of reactions 2 and 3 is reaction 1, with the additional result that inorganic phosphate is taken up during the course of the reaction and ATP synthesised.

Hydrogen and carbon dioxide formation

The phosphoclastic split described above (reaction 2), releases formic acid as a product of pyruvic acid breakdown. Formic acid is further broken down to hydrogen and carbon dioxide by an enzyme called formic hydrogenlyase,

$$H.COOH \xrightarrow[\text{hydrogenlyase}]{\text{Formic}} H_2 + CO_2 \quad \ldots\ldots\ldots(4)$$

The hydrogen formed during fermentation is all produced as a result of this reaction, though, as we shall see later, the phosphoclastic splitting of pyruvic acid is not the sole source

of formic acid as precursor of hydrogen. Formic hydrogenlyase is a reversible enzyme, so that synthesis of formic acid will occur in the presence of hydrogen and carbon dioxide. In the absence of any side-reactions, the final result of formic hydrogenlyase action is to produce an equilibrium mixture of formic acid, hydrogen, and carbon dioxide. Formic hydrogenlyase is an iron-activated enzyme, and if the organism is grown in an iron-deficient medium, the formation of the enzyme is prevented and, as a result, no gas is liberated during the fermentation of glucose, formic acid accumulating instead (see p. 83).

Lactic acid formation

Esch. coli possesses the same series of enzymes as those involved in the production of lactic acid by muscle. It possesses lactic dehydrogenase which will bring about the reduction of pyruvic acid to lactic acid in the presence of a H-donator, which is itself supplied by the presence of reduced coenzyme I. The series of reactions involved in the fermentation of glucose includes the oxidation of glyceraldehyde-phosphate to phosphoglyceric acid by triose-phosphate dehydrogenase, the oxidation involving the reduction of coenzyme I. If, however, the fermentation substrate is pyruvic acid instead of glucose, then triose-phosphate dehydrogenase is not involved and coenzyme I is not reduced. This is probably linked with the facts that whereas lactic acid forms approximately 50 per cent. of the products of glucose fermentation, it forms only about 5 per cent. of the products when pyruvic acid is the fermentation substrate. The main reaction giving rise to lactic acid is presumably therefore:

$$\begin{array}{l} \mathrm{CHO} \\ \mathrm{CHOH} \\ \mathrm{CH_2OPO_3H_2} \end{array} + \mathrm{H_2O} + \begin{array}{l} \mathrm{CH_3} \\ \mathrm{CO} \\ \mathrm{COOH} \end{array} \longrightarrow \begin{array}{l} \mathrm{COOH} \\ \mathrm{CHOH} \\ \mathrm{CH_2OPO_3H_2} \end{array} + \begin{array}{l} \mathrm{CH_3} \\ \mathrm{CHOH} \\ \mathrm{COOH} \end{array} \quad \ldots\ldots(5)$$

the oxidation of glyceraldehyde-phosphate being linked to the reduction of pyruvic acid by coenzyme I acting as H-carrier

(see p. 127). However, lactic acid is still formed to some extent from pyruvic acid, and it has been suggested that formic acid will react with pyruvic acid to give lactic acid according to the equation:

$$CH_3.CO.COOH + H.COOH \longrightarrow CH_3.CHOH.COOH + CO_2 \quad \ldots\ldots (6)$$

It is found experimentally that a molecule of CO_2 is formed for every molecule of lactic acid produced. As a result of this, the fermentation gases contain more carbon dioxide than hydrogen, the amount of carbon dioxide in excess of the hydrogen being equivalent to the lactic acid formed. The experimental evidence in support of reaction 6 is not, however, convincing at present. It has also been shown that the amount of lactic acid produced is increased by increasing the carbon dioxide present during the fermentation. Fermentation studies carried out in the presence of carbon dioxide containing isotopic C^{13} ("heavy carbon") have shown that $C^{13}O_2$ is fixed during the fermentation, and that some of this fixed-CO_2 appears in the —COOH group of lactic acid.

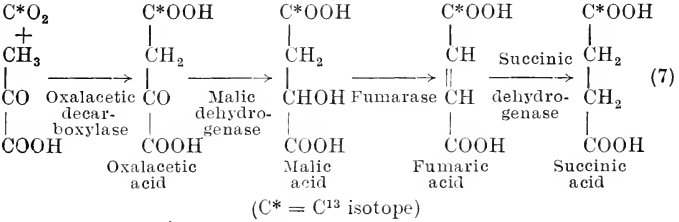
In the case of the *Gonococcus* and *S. faecalis* it has been shown that lactic acid can be formed from pyruvic acid by a hydrolytic reaction (or dismutation):

$$2CH_3.CO.COOH + H_2O = CH_3.CHOH.COOH + CH_3.COOH + CO_2.$$

Succinic acid formation

Succinic acid is formed to a variable extent during the fermentation of either glucose or pyruvic acid by *Esch. coli*, *Aerobacter aerogenes*, *Propionibacteria*, and other organisms. For many years its formation constituted a puzzle, as it was by no means obvious how a substance containing 4 carbon atoms could be derived from a 6-carbon sugar or a 3-carbon triose or pyruvic acid. Elsden of the Cambridge School showed that succinic acid formation depends upon the presence of carbon dioxide during fermentation, so that increasing the CO_2 tension results in increased succinic acid production or,

alternatively, rigid removal of carbon dioxide during fermentation results in suppression of succinic acid production. This suggests that carbon dioxide is assimilated during the fermentation and that the ultimate fate of the assimilated carbon dioxide is to form succinic acid. This suggestion has been investigated in detail by Prof. Werkman and his colleagues at Iowa State College. They showed that if pyruvic acid is fermented in the presence of carbon dioxide containing isotopic C^{13}, then some of the heavy-carbon is assimilated and appears in one of the —COOH groups of succinic acid. They suggest the following scheme for the reaction:—



(7)

($C^* = C^{13}$ isotope)

CO_2 is first fixed by combination with pyruvic acid to form oxalacetic acid. The enzyme involved in this fixation process is oxalacetic acid decarboxylase working in reverse. Oxalacetic acid is then reduced to malic acid by malic dehydrogenase in reverse. Malic acid is converted to fumaric acid by the action of fumarase and, finally, fumaric acid is reduced to succinic acid by succinic dehydrogenase acting in reverse. All these enzymes have been demonstrated in the bacteria concerned, and it is probable that the "Wood-Werkman scheme," as this is usually called, is responsible for part, at least, of the succinic acid formation in these organisms.

This scheme accounts for succinic acid formation by the fixation of carbon dioxide, but studies involving isotopic CO_2 show that, although some of the succinic acid arises in this fashion, the total formation of succinic acid cannot be accounted for in this way. Werkman and his colleagues have now demonstrated that there is a second method whereby

succinic acid is formed in bacterial fermentation and that is by dehydrogenation of acetic acid,

$$\begin{array}{l} CH_3.COOH \\ \quad + \\ CH_3.COOH \end{array} \rightleftharpoons \begin{array}{l} CH_2.COOH \\ | \\ CH_2.COOH \end{array} + 2H \quad \ldots\ldots(8)$$

In some cases, but not all, this reaction is easily reversed, and acetic acid itself can be formed by reductive breakdown of succinic acid. These reactions have again all been demonstrated by application of techniques involving heavy-carbon.

THE FERMENTATION OF PYRUVIC ACID BY *AEROBACTER AEROGENES*

Formation of acetylmethylcarbinol, etc.

Aerobacter aerogenes is an organism very similar in many properties to *Esch. coli*, but systematically differentiated from the latter by a positive " Voges-Proskauer test " (Table II). This test consists of adding strong alkali to a 24 hours old culture of the organism in glucose-peptone, and a positive reaction is shown by the development of a pink colour near the surface of the medium after 24-48 hours. The colour starts to develop at the surface of the medium and slowly spreads down into the liquid. The chemistry of the colour reaction is complex and is due to a reaction between diacetyl, $CH_3.CO.CO.CH_3$, and substances in the medium containing a guanidino-group. To speed up the test it is usual nowadays to add a trace of creatinine to the treated medium when, if positive, the colour develops within a short time. Diacetyl is produced by atmospheric oxidation of acetylmethylcarbinol (acetöin), $CH_3.CO.CHOH.CH_3$, which is a fermentation product formed from glucose by this organism.

Glucose is fermented by the organism to pyruvic acid, as usual, and *Aerobacter aerogenes* then attacks pyruvic acid in two ways.

(*a*) By the phosphoclastic split to acetic and formic acids in exactly the same way as *Esch. coli*.

(*b*) By decarboxylation and condensation of two molecules of pyruvic acid to form acetylmethylcarbinol,

$$2CH_3.CO.COOH \longrightarrow CH_3.CO.CHOH.CH_3 + 2CO_2 \quad \ldots . (9)$$

Which of the reactions predominates depends upon the *p*H of the growth medium. Acetylmethylcarbinol is formed only when growth occurs at an acid *p*H and the *p*H of optimum activity of the enzyme involved lies between 4·0 and 5·5. The enzyme has been obtained in a cell-free state, and is developed within the cell only when growth occurs at an acid *p*H; if the *p*H is maintained at an alkaline value by the addition of alkali throughout growth, so that the fermentation acidity is neutralised, then the acetylmethylcarbinol enzyme is not formed and the fermentation of the organism is consequently essentially similar to that of *Esch. coli*. It is not possible to convert *Aerobacter aerogenes* into *Esch. coli* by continued growth in alkaline media, as immediately growth is resumed in acid conditions acetylmethylcarbinol formation again takes place.

Reaction 9 thus occurs under acid environmental conditions. Since it involves the conversion of two molecules of acid into one molecule of a neutral substance, it acts as a neutralisation mechanism (see p. 71), coming into action when the growth environment becomes acid. This fact is further utilised as a method of differentiating between *Aerobacter* and *Escherichia*. If the two organisms are cultivated in a medium containing a small amount of glucose, then both will ferment the glucose with the formation of acid. However, the acetylmethylcarbinol formation by *Aerobacter* will result in the neutralisation of some of this acid and, as long as the glucose is not present in excess, the final *p*H will be lower in the *Escherichia* culture than in the *Aerobacter* culture. Consequently, if we put up our cultures in peptone containing 0·2 per cent. glucose and methyl red as indicator, we find that *Esch. coli* will turn the indicator red in the course of its growth, while *Aerobacter* will not; the *Esch. coli* is therefore said to be "methyl red positive."

Some strains of *Aerobacter* can effect a reduction of acetylmethylcarbinol to 2.3.butylene glycol:

$$CH_3.CO.CHOH.CH_3 + 2H \rightleftharpoons CH_3.CHOH.CHOH.CH_3 \quad (10)$$

This fermentation product is of considerable commercial interest as it is a comparatively simple matter to convert it by chemical means to butadiene, $CH_2{=}CH{-}CH{=}CH_2$, which is one of the materials used to produce synthetic rubber by polymerisation. Acetylmethylcarbinol also undergoes oxidation by oxygen to diacetyl $CH_3.CO.CO.CH_3$, which is the substance imparting the "buttery" smell to butter.

Acetylmethylcarbinol is produced by species of *Pseudomonas* and *Bacillus* as well as by *Aerobacter*.

THE FERMENTATION OF PYRUVIC ACID BY *PROPIONIBACTERIA*

Certain bacteria found in Gruyère and Emmentaler cheeses produce propionic acid amongst their fermentation products. These *Propionibacteria* ferment pyruvic acid to form propionic acid, and since they also reduce lactic acid to this same product, it has been suggested in the past that lactic acid forms an intermediate stage in the reduction of pyruvic acid to propionic acid. However, Barker and Lipmann have found that the decomposition of lactate is inhibited by sodium fluoride at a concentration considerably less than that required to prevent the formation of propionic acid from pyruvic acid. This indicates that lactic acid cannot act as an intermediate in the latter reaction, but that the following scheme is involved.

$$CH_3.CO.COOH \xrightarrow{2H} X \xrightarrow{2H} CH_3.CH_2.COOH$$

$$\uparrow \text{NaF}$$

$$CH_3.CHOH.COOH$$

where the nature of X is unknown.

The *Propionibacteria* form succinic acid from pyruvic acid by the fixation of carbon dioxide in the same way as that

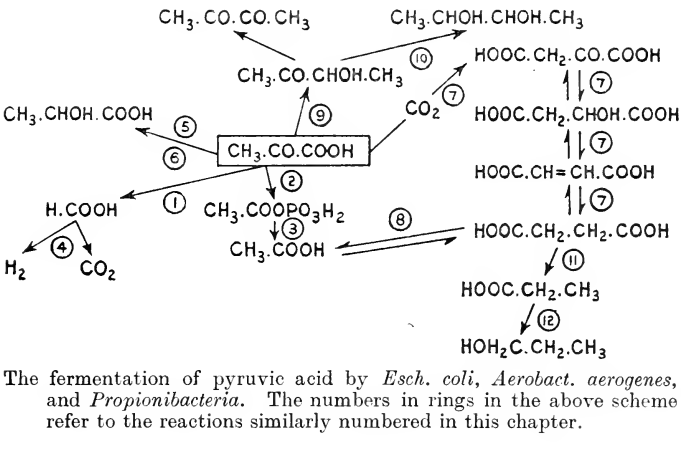
described for *Esch. coli*. Werkman and his colleagues have shown that if CO_2 containing C^{13} is used then the heavy-carbon is fixed by the organism and appears in the —COOH group of succinic acid and also of propionic acid. This suggests that propionic acid arises from the same initial reaction, involving CO_2 fixation, as succinic acid. It is now known that the organism possesses a succinic decarboxylase which removes CO_2 from succinic acid, to form propionic acid.

$$\begin{array}{ccccccccccc} CO_2 & & COOH & & COOH & & COOH & & COOH & & COOH \\ + & & | & & | & & | & & | & & | \\ CH_3 & & CH_2 & & CH_2 & & CH & & CH_2 & & CH_2 \\ | & \rightleftharpoons & | & \rightleftharpoons & | & \rightleftharpoons & \| & \rightleftharpoons & | & \longrightarrow & | \\ CO & & CO & & CHOH & & CH & & CH_2 & & CH_3 \\ | & & | & & | & & | & & | & & + \\ COOH & & COOH & & COOH & & COOH & & COOH & & CO_2 \end{array} \quad \ldots.(11)$$

Some strains of these organisms carry out a further reduction of propionic acid to *n*-propyl alcohol,

$$CH_3.CH_2.COOH + 2H_2 \longrightarrow CH_3.CH_2.CH_2OH + H_2O \quad \ldots(12)$$

and when $C^{13}O_2$ is used in such a fermentation, C^{13} appears in the —COOH groups of succinic and propionic acids and also in the $—CH_2OH$ of propyl alcohol.



The fermentation of pyruvic acid by *Esch. coli*, *Aerobact. aerogenes*, and *Propionibacteria*. The numbers in rings in the above scheme refer to the reactions similarly numbered in this chapter.

MISCELLANEOUS FERMENTATION REACTIONS

In the preceding sections we have traced the formation and fate of pyruvic acid in various fermentations. It is not possible, however, to derive all fermentation products from pyruvic acid, and we must now consider the production of some of these substances. The point is experimentally tested by comparing the fermentation products of an organism when, first, glucose and, second, pyruvic acid is used as fermentation substrate. Thus the products of the fermentation of glucose by *Esch. coli* are H_2, CO_2, ethyl alcohol, formic acid, acetic acid, lactic acid, and succinic acid, but if pyruvic acid is the substrate then no ethyl alcohol is formed and very much less lactic acid.

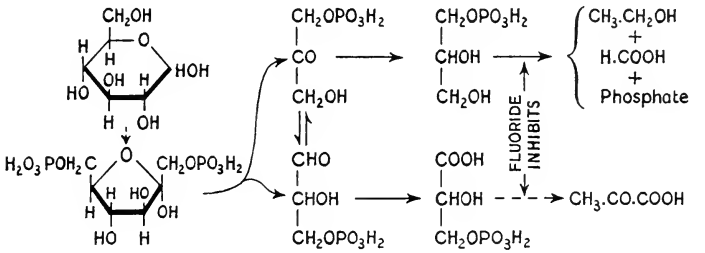
Ethyl alcohol formation

In the alcoholic fermentation of yeast (*Saccharomyces cerevisiae*), alcohol is derived from pyruvic acid by the action of carboxylase which decarboxylates pyruvic acid to acetaldehyde:

$$CH_3.CO.COOH \xrightarrow{\text{Carboxylase}} CH_3.CHO + CO_2,$$

and acetaldehyde is then reduced to alcohol by alcohol dehydrogenase working in reverse. The coliform organisms do not, however, possess carboxylase, and the presence of this enzyme in bacteria has yet to be demonstrated. Ethyl alcohol does not arise from pyruvic acid in these organisms.

If we return to the fermentation of glucose, as described on p. 128, we find that the formation of pyruvic acid was traced from hexosediphosphate through glyceraldehyde-phosphate and phosphoglyceric acid. The oxidation of glyceraldehyde-phosphate to phosphoglyceric acid forms half of an oxido-reduction reaction, which is completed, for the other half, by the reduction of dihydroxyacetone-phosphate to α-glycerophosphate. If fluoride is added to the fermentation system, then the reaction is stopped at this point and a mixture of phosphoglyceric acid and α-glycerophosphate remain as the products.

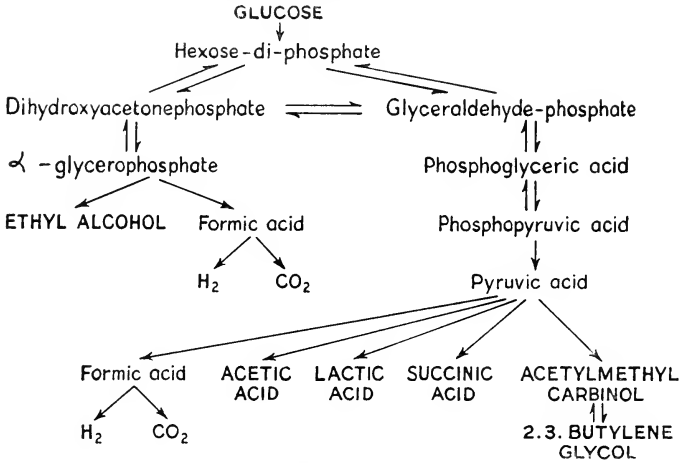


In the absence of fluoride, phosphoglyceric acid is fermented to pyruvic acid, as described, and α-glycerophosphate is fermented with the formation of ethyl alcohol and formic acid. Dihydroxyacetone-phosphate and glyceraldehyde-phosphate form an equilibrium mixture; consequently the proportions of ethyl alcohol to pyruvic acid formed will depend upon the reaction velocities of the various intermediate reactions involved.

It will be noted that the oxidation of glyceraldehyde-phosphate can be coupled *either* with a reduction of dihydroxy-acetone-phosphate *or* with the reduction of pyruvic acid to lactic acid. Since pyruvic acid will not accumulate until the glycolysis cycle has proceeded through all the intermediate steps, it follows that the reduction of dihydroxyacetone-phosphate will occur predominantly during the starting-up of the cycle and so can be looked upon as a " starter reaction " which enables the cycle to get under way and consequently to produce the main H-acceptor, pyruvic acid.

Investigations using isotopic-carbon have indicated that, during the fermentation of glucose, ethyl alcohol can also be formed by the reduction of acetic acid. Ethyl alcohol is not formed during the fermentation of pyruvic acid but it is possible that the reduction of acetic acid requires reduced coenzyme I as H-donor, and this, in turn, requires the triose-phosphate dehydrogenase system, as in the case of lactic acid formation. This second method of alcohol formation has not yet been confirmed by other techniques.

It is now possible to outline the intermediate reactions involved in the formation of all the fermentation products of the *coli-aerogenes* group of organisms:



Acetone-butanol fermentation

The fermentation of maize meal or molasses by *Cl. acetobutylicum* became of importance in the war of 1914-18 as it was then the most satisfactory method of making acetone on a commercial scale. Since that time the synthesis of acetone by a cheaper chemical method has been worked out and the fermentation method is no longer of such industrial importance. A further product of the fermentation is butyl alcohol (butanol) which is now required on a large scale as a paint and lacquer solvent. As a result of the commercial value of the products, the fermentation has received considerable attention which has not, as yet, succeeded in unravelling all the intermediate reactions. The research was hampered by the fact that, until recently, it has not been possible to prepare washed suspensions of the organism in an active state, but this difficulty has now been largely overcome by the use of concentrated suspensions, rather than washed suspensions, and a certain amount of knowledge of the course of the fermentation has been obtained.

In the commercial process the raw fermentation substrate is usually maize meal, and the organism attacks the starch of maize meal by the production of two extracellular enzymes, one of which is an amylase which breaks the starch down to maltose, and the other is a maltase which hydrolyses the maltose to glucose. The fermentation of glucose by *Cl. acetobutylicum* gives rise to the formation of hydrogen, carbon dioxide, acetic and butyric acids, ethyl and butyl alcohols, and acetone; the fermentation of pyruvic acid gives rise mainly to hydrogen, carbon dioxide, and acetic acid, with traces of butyric acid, ethyl alcohol, and acetone. The proportions in the two cases are given in Table XIII, where the results are expressed as molecules of product per mol. glucose or two mols. pyruvic acid (equivalent to 1 mol. glucose) fermented.

TABLE XIII

PRODUCTS OF *Cl. acetobutylicum* FERMENTATION

Product	Substrate-glucose mols./mol.	Substrate-pyruvate mols./2 mols.
Hydrogen gas	1·87	1·88
Carbon dioxide	2·46	2·08
Butyl alcohol	0·6	0·01
Ethyl alcohol	0·006	0·05
Acetone	0·14	0·08
Acetic acid	0·125	1·52
Butyric acid	0·04	0·1

Investigations of this fermentation have been concerned mainly with the production of the commercially valuable substances, acetone and butyl alcohol. If the formation of the various products is followed at intervals during growth in a glucose-containing medium, then we find that the appearance of these substances varies with the time as shown in Fig. 11.

In the early stages of growth, while the *p*H is falling rapidly, acetic and butyric acids are formed together with hydrogen and carbon dioxide, but no acetone or butyl alcohol. Later in

the age of the culture, when the *p*H has fallen to about 4·5, acetone and butyl alcohol begin to appear; their appearance is associated with a corresponding disappearance of acetic and butyric acids and a small rise in *p*H. It would seem that the formation of acetone and butyl alcohol, involving the formation of neutral substances from acids, is a neutralisation mechanism which is brought into play when the environmental *p*H becomes strongly acid. Their formation can thus be regarded as a mechanism in the same class as that of

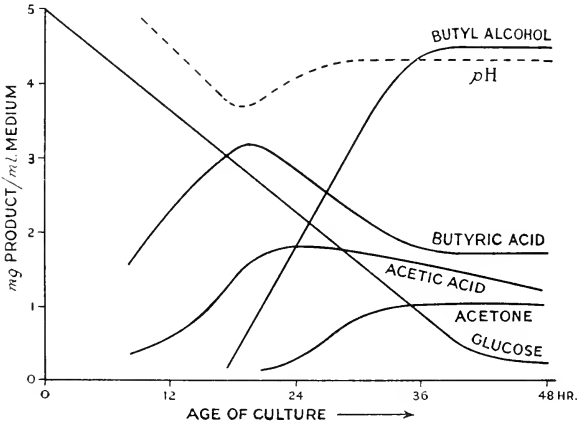


Fig. 11. Course of fermentation of glucose by *Cl. acetobutylicum* [after Davies and Stephenson, *Biochem. J.*, 1941, **35**, 1323].

acetylmethylcarbinol formation in *Aerobacter aerogenes*, and of the production of amines from amino-acids by some strains of *Esch. coli*. It would appear from Fig. 11 that the precursors of acetone and butyl alcohol in the medium are acetic and butyric acids respectively, and it has been shown that the addition of acetate to the fermentation mixture results in a marked increase in the production of acetone. At first it was thought that a direct reduction of the acids by fermentation hydrogen might take place, but it is fairly certain now that this is not the case and that acetic and butyric acids undergo

further metabolic changes before giving rise to acetone and butyl alcohol.

Acetone is produced by the decarboxylation of acetoacetic acid:

$$CH_3.CO.CH_2.COOH \xrightarrow[\text{decarboxylase}]{\text{Acetoacetic}} CH_3.CO.CH_3 + CO_2,$$

and the acetoacetic decarboxylase, which has been obtained in a cell-free condition from the organism, is formed within the cells only when the environmental *p*H has fallen to a low value—the growth conditions stimulating its formation being similar to those stimulating the formation of the amino-acid decarboxylases in other bacteria. Accumulation of acetoacetic acid in the medium can be demonstrated by means of a colour reaction, and the presence of the acid can be shown towards the end of the growth of the culture when acetone formation is taking place. There is little doubt but that acetic acid is reduced to acetone through acetoacetic acid, but it is not known how acetoacetic acid is formed from acetic acid. Recent investigations, using isotopic-C compounds, suggest that acetic acid is also the precursor of butyric acid and butyl alcohol.

Iso-propyl alcohol, $CH_3.CHOH.CH_3$, is formed by the fermentation of *Cl. butylicum*, an organism closely related to *Cl. acetobutylicum*. Isotopic-C studies again indicate that the iso-propyl alcohol is formed by reduction of acetone, which is formed by the organism in the same way as by *Cl. acetobutylicum*, but the acetone in this case is reduced to the corresponding alcohol so rapidly that it does not accumulate and appear as a fermentation end-product.

Fatty Acid Synthesis

The synthesis of butyric acid has been investigated in detail by Barker and his colleagues for the case of *Cl. kluyverii*. This is a strict anaerobe, isolated from mud, which cannot utilise glucose but requires ethyl alcohol and a fatty acid such as acetate for growth. It obtains its energy by metabolism of these substances resulting in the synthesis of higher fatty acids;

when growing on alcohol and acetate it synthesises butyric and caproic acids with the liberation of some hydrogen. If acetic acid, isotopically labelled (C*) in the position CH_3C^*OOH, is added to the culture, the resulting acids are labelled in the positions $CH_3.C^*H_2.CH_2.C^*OOH$ (butyric) and $CH_3.C^*H_2.CH_2.C^*H_2.CH_2.C^*OOH$ (caproic) indicating that they are formed by condensation of acetate molecules. Cell-free enzyme preparations have been made which will accomplish the synthesis of butyric and caproic acids from ethyl alcohol and acetate under anaerobic conditions. Analysis of the reactions involved shows that the alcohol is first oxidised to acetaldehyde and acetylphosphate with the liberation of hydrogen, and this hydrogen is then utilised to reduce (acetylphosphate + acetate) to butyric acid. The over-all reactions can be represented:—

1. $2CH_3.CH_2OH + H_3PO_4 \rightarrow CH_3.CHO + CH_3.COOPO_3H_2 + 3H_2$
2. $CH_3.COOPO_3H_2 + CH_3.COOH + 2H_2 \rightarrow CH_3.CH_2.CH_2.COOH + H_3PO_4 + H_2O$

The initial step involved in the condensation of acetylphosphate and acetate has not yet been clarified. The work on acetone production, outlined above, suggests that acetoacetic acid might be concerned but if this substance is added to the enzyme preparation it is either split irreversibly to acetylphosphate and acetate or, in the presence of hydrogen, reduced to β-hydroxybutyric acid and does not give rise to butyric acid under any condition tested. A number of other possible intermediate substances have now been tested and the only one which will give rise to butyric acid is vinyl acetate, $CH_2 = CH.CH_2.COOH$, but isotope studies indicate that it is not, in fact, involved in the production of butyric acid from acetylphosphate and acetate. The present situation concerning the synthesis of butyric acid by *Cl. kluyveri* can be summarised diagrammatically as follows:—

$$\begin{array}{ccccc} & CH_3.CH_2OH & & CH_2:CH.CH_2.COOH & \\ & \downarrow & & \downarrow 4H & \\ CH_3.COOH + & CH_3.COOPO_3H_2 & \longrightarrow ? \longrightarrow & CH_3.CH_2.CH_2.COOH & + H_3PO_4 \\ & \uparrow & & 2H & \\ & CH_3.CO.CH_2.COOH & \longrightarrow & CH_3.CHOH.CH_2.COOH & \end{array}$$

Caproic acid may be formed by a further condensation of acetylphosphate with butyric acid:—

$$CH_3.CH_2.CH_2.COOH + CH_3.COOPO_3H_2 + 4H \rightarrow CH_3.CH_2.CH_2.CH_2.CH_2.COOH + H_3PO_4 + H_2O.$$

FOR FURTHER READING

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CHAPTER VIII

PROVISION OF ENERGY: OXIDATION

In the last chapter we dealt in detail with the anaerobic decomposition of carbohydrates. In this chapter we shall proceed to study the liberation of energy by oxidative processes. As explained in Chap. III, oxidation does not necessarily involve molecular oxygen since any substance AH_2 can be oxidised by the general oxido-reduction reaction:

$$AH_2 + B \rightleftharpoons A + BH_2.$$

Oxygen can take the place of the hydrogen-acceptor B. Oxidation reactions thus do not depend upon the presence of atmospheric oxygen and can be carried out by anaerobic as well as aerobic organisms. However, where the organism is living an aerobic existence and a substrate AH_2 can be oxidised directly, or indirectly through a chain of reactions, by oxygen, the accumulation of reduced products is avoided and the provision of energy by such complete oxidation is obviously greater than by a fermentation process. Consequently a facultative anaerobe grows more efficiently in air than anaerobically.

The oxidation of carbon substances is too wide and complex a subject to deal with in detail here, and we shall restrict the discussion to the mechanisms employed by typical examples of aerobic, facultatively anaerobic, and anaerobic bacteria to carry out key oxidations centring on alcohol as substrate.

OXIDATIONS IN *ACETOBACTER*

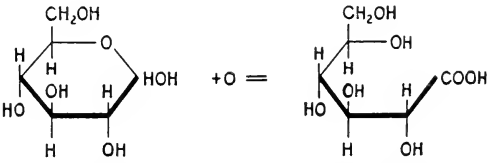
The commercial production of acetic acid as vinegar has been based for many years on the power of certain strictly aerobic bacteria, *Acetobacter*, to oxidise ethyl alcohol. The process has been commonly called "acetic fermentation,"

though it cannot strictly be called a fermentation if we adhere to the usual definition of fermentation as an anaerobic process. The biological nature of the process was demonstrated by Pasteur in 1862-4, and since then many organisms of this genus have been isolated from vinegar vats, etc., and have the property of oxidising alcohols to acid. Acetobacter are highly aerobic and carry out three main types of oxidation:

1. Oxidation of primary alcohols to the corresponding acids:

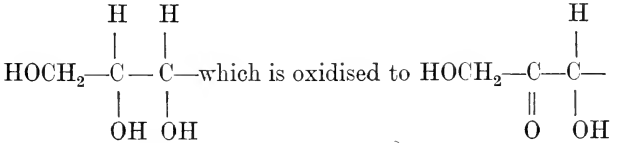
$$CH_3.CH_2OH + O_2 = CH_3.COOH + H_2O.$$

2. Oxidation of aldehydes and aldohexoses to the corresponding acids:

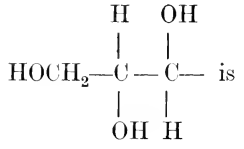


GLUCOSE GLUCONIC ACID

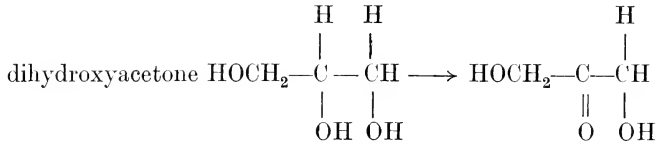
3. Oxidation of certain secondary alcohols to the corresponding ketones. The nature of the secondary alcohols was studied in detail by Bertrand in a classical work on *Acetobacter xylinum*. By studying a large number of secondary alcohols, he showed that only those possessing a specific stereochemical configuration are attacked. Using the old type of straight chain formula, this specific group is



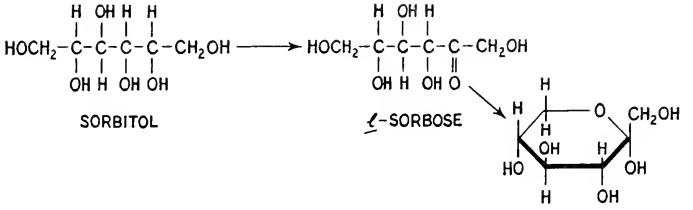
and the —OH group oxidised must be in the β-position and adjacent to another —OH group. Thus an alcohol with the structure



is not attacked. Glycerol is oxidised to dihydroxyacetone



A strain of *Acetobacter* is used to oxidise sorbitol to sorbose as one of the steps in the commercial synthesis of vitamin C.



OXIDATION OF ALCOHOL BY *ESCH. COLI*

In the case of the oxidation of ethyl alcohol by *Esch. coli* the enzyme system has been analysed and the various components identified. The alcohol is oxidised to acetaldehyde in the first place:

$$CH_3.CH_2OH + O \longrightarrow CH_3.CHO + H_2O \ldots . \quad (1)$$

The enzyme concerned is alcohol dehydrogenase which transfers hydrogen from alcohol to coenzyme I,

$$CH_3.CH_2OH + \text{coenzyme I} \xrightarrow[\text{dehydrogenase}]{\text{Alcohol}} CH_3.CHO + \text{reduced co. I} \quad (2)$$

Reduced coenzyme then reacts with the cytochrome system

of the organism, the transfer of the hydrogen being catalysed by diaphorase (see substrate Type S_3, Table III).

$$\text{Reduced coenzyme I} + \text{Cytochrome} \xrightarrow{\text{Diaphorase}} \text{Coenzyme} + \text{Reduced cytochrome} \quad (3)$$

and the final link with atmospheric oxygen is made by cytochrome oxidase catalysing the transfer of hydrogen from reduced cytochrome to combine with atmospheric oxygen, forming water. The complete series of reactions have the over-all results of Reaction I. Acetaldehyde is further oxidised, presumably to acetic acid in the first place, although this does not seem to have been proved:

$$CH_3.CHO + O \longrightarrow CH_3.COOH \quad \ldots\ldots\ldots (4)$$

Mammalian tissues carry out a similar oxidation through the action of aldehyde oxidase which is a flavoprotein and catalyses the oxidation of its substrates by atmospheric oxygen without the intermediary action of other carriers (substrate Type S_1, Table III). The oxidation has not been studied in detail in *Esch. coli*. Acetic acid is also formed from pyruvic acid and the enzyme concerned, pyruvic oxidase, has been studied in extracts of *L. delbreuckii*. If the enzyme preparation is purified and dialysed, it will attack pyruvic acid only in the presence of thiamindiphosphate and inorganic phosphate, and the products of the reaction are acetyl phosphate and carbon dioxide:

$$O + CH_3.CO.COOH + H_3PO_4 = CH_3.COOPO_3H_2 + CO_2 + H_2O \ldots\ldots (5)$$

If adenosine-di-phosphate (ADP) is added as phosphate-acceptor, then the acetyl phosphate gives up its phosphate to form adenosine-tri-phosphate and acetic acid:

$$CH_3.COOPO_3H_2 + ADP = CH_3.COOH + ATP \ldots\ldots (6)$$

The over-all reaction therefore involves an oxidation of pyruvic acid to acetic acid and the synthesis of ATP from inorganic phosphate. This reaction is therefore called an

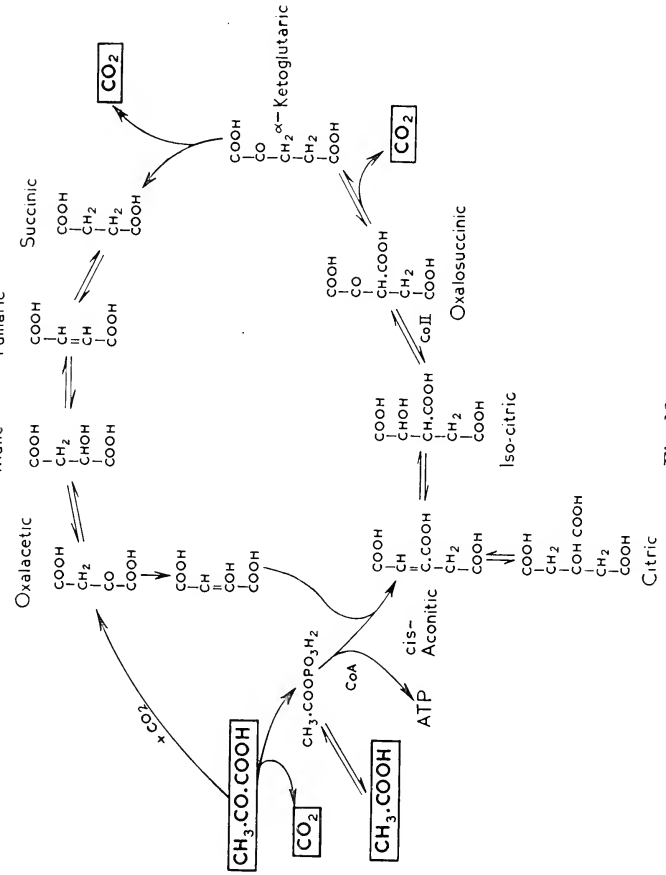

Malic
Fumaric
Succinic
Oxalacetic
α-Ketoglutaric
Oxalosuccinic
Iso-citric
cis-Aconitic
Citric
CO2
CH3.CO.COOH
CH3.COOH
CH3.COOPO3H2
CoA
ATP
CoII

Fig. 12.

" oxidative phosphorylation " and it is clear that it has resulted in the production of energy-rich ATP. This reaction demonstrates how oxidation energy is made available for synthetic purposes by the cell; the energy released by the oxidation accumulates in ATP just as it did in the case of the oxidation of glyceraldehyde-phosphate in the fermentation cycle. Both fermentation and oxidation therefore have the same end result in the synthesis of energy-rich ATP bonds within the cell.

Under aerobic conditions, acetic acid itself is oxidised by *Esch. coli*:

$$CH_3.COOH + 2O_2 = 2CO_2 + 2H_2O \quad \ldots\ldots\ldots (7)$$

The enzymic processes involved in this reaction in bacteria are not yet clear. In animal tissues a similar type of oxidation takes place through a complex cycle known as the " Krebs " or " citric acid cycle." This cycle involves a complex of many enzymes intervening between acetate (or acetyl phosphate) and the cytochrome system. It has two main results: (1) it splits up the liberation of oxidative energy into small steps instead of a single large outburst, and (2) since a number of steps involve uptake of inorganic phosphate (not shown in Fig. 12) followed by synthesis of ATP, it makes energy available to the cell in the form of energy-rich phosphate bonds. The steps and enzymes involved in the cycle are set out in Fig. 12. Although this cycle has now been well established for animal tissues, there is considerable doubt whether it exists in bacteria. That part of the cycle connecting pyruvic acid through oxalacetic acid to succinic acid is the same as that which has been discussed in Chap. VII in the section on CO_2 fixation; it has recently been shown that acetyl phosphate will condense with oxalacetate in the presence of coenzyme A to yield citric acid in *Esch. coli*. On the other hand, *Esch. coli* is differentiated from *Aerobacter* in that it cannot attack citric acid, while in *Azotobacter* it is found that the rate of oxidation of acetate is greater than that of any of the intermediate substances in the postulated cycle. For the present it must suffice to

record the cycle as known in other tissues and to point out that, although there is evidence accumulating that some such system does exist in bacteria, its nature and occurrence in any bacterium has yet to be proved.

The oxidation process makes ATP available and this, in turn, makes energy available for synthetic purposes. If we follow the oxygen uptake during the oxidation of acetate by *Esch. coli*, we find that the amount of gas taken up corresponds to 60-75 per cent. of that required by the above equation for complete oxidation. If the residual substrate is estimated, we find that all the acetic acid has disappeared, although the oxygen consumption does not correspond to 100 per cent. oxidation. The portion of the acetic acid which has not been oxidised is assimilated and incorporated in the cells by what is called a process of "oxidative assimilation." If we assume that the material assimilated by the cells is of the nature (HCOH), then the true equation for the oxidation is:

$$2CH_3.COOH + 3O_2 \longrightarrow (HCOH) + 3CO_2 + 3H_2O.$$

If the oxidation is carried out in the presence of sodium azide or dinitrophenol the oxidative assimilation is prevented and the oxygen consumption then corresponds to quantitative oxidation according to Equation 7.

OXIDATION BY STRICT ANAEROBES

The methane that arises from stagnant and putrescent pools is produced by bacterial action. The organisms responsible belong to the genus *Methanobacter*, and their activities have been studied in detail by Barker and his colleagues. *Methanobacter omelianskii*, like other organisms of this group, is a strict anaerobe, and obtains energy by the oxidation of alcohols. As it is a strict anaerobe it cannot utilise oxygen for the oxidation process, but carries out an oxidation-reduction process in which the H-acceptor is carbon dioxide, which is reduced to methane according to the equation:

$$2CH_3.CH_2OH + CO_2 \longrightarrow 2CH_3.COOH + CH_4.$$

The organism will not attack methyl alcohol but will grow on ethyl alcohol, when the rate of growth bears a linear relation to the acetic acid production, and the greater part of the carbon of the organism is derived from the acetate so formed. Proof that methane arises from carbon dioxide was obtained by carrying out the oxidation of alcohol in the presence of carbon dioxide enriched with isotopic-C, when it was found that the carbon dioxide was converted to methane and the bulk of the isotopic-carbon appeared in the methane produced, although small amounts were assimilated by the organism.

The strictly anaerobic Clostridia obtain their energy in many cases by oxido-reduction reactions. In some cases the hydrogen donator and acceptor are both amino-acids, so that a reaction occurs in which one amino-acid is oxidised to the corresponding keto-acid, while the other is reduced to the corresponding fatty acid, both amino-acids becoming deaminated:

$$\begin{array}{l} \mathrm{R} \\ | \\ \mathrm{CHNH_2} \\ | \\ \mathrm{COOH} \end{array} + \mathrm{H_2O} + \begin{array}{l} \mathrm{X} \\ | \\ \mathrm{CHNH_3} \\ | \\ \mathrm{COOH} \end{array} \longrightarrow \begin{array}{l} \mathrm{R} \\ | \\ \mathrm{CO} \\ | \\ \mathrm{COOH} \end{array} + 2\mathrm{NH_3} + \begin{array}{l} \mathrm{X} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{COOH} \end{array}$$

This reaction is called the Stickland reaction and is discussed in further detail in Chap. IX.

Alcohol is a hydrogen donator in the case of *Cl. kluyverii* studied by Barker and his co-workers (pp. 145-7). This organism cannot attack glucose or pyruvic acid but obtains its energy by the metabolism of ethanol and acetate. The growth requires CO_2, and isotopic studies have shown that the amount of CO_2 assimilated is proportional to the amount of acetate metabolised, but that 70 per cent. of the carbon of the CO_2 appears in the cellular material and none in the other products. The main products of the metabolism of the ethanol and acetate are butyric and caproic acids, but gaseous hydrogen is also formed and this arises from oxidation of ethanol:

$$\mathrm{CH_3CH_2OH + H_2O \rightarrow CH_3.COOH + 2H_2.}$$

In this case CO_2 is utilised for cell synthesis, the energy being provided by fatty acid metabolism. However, in other cases amongst the Clostridia, the CO_2 can act as hydrogen acceptor as well as a source of cell-carbon, *e.g.* Wieringa isolated an organism from mud which reduces CO_2 to acetic acid in the presence of gaseous hydrogen as H-source:

$$2CO_2 + 4H_2 \rightarrow CH_3.COOH + 2H_2O.$$

FOR FURTHER READING

"Microbial Assimilations," Clifton, C. E., *Advances in Enzymology*, 1946, **6**, 269.

"The Intermediate Stages in the Biological Oxidation of Carbohydrate," Krebs, H. A., *Advances in Enzymology*, 1943, **3**, 191.

Papers:

1. Bertrand, G., *Ann. Chim. et Phys.*, **8**, 181 (*Acetobacter*).

2. Still, L., *Biochem. J.*, 1940, **34**, 1177 (*Esch. coli*).

3. Barker, H. A., *J. Biol. Chem.*, 1941, **137**, 153 (*Methanobacter*).

CHAPTER IX

BREAKDOWN OF NITROGENOUS MATERIAL

The synthesis of protein is one of the main reactions involved in the growth of the bacterial cell. In Chap. V we were largely concerned with the nature of the bricks from which the cell builds its protein and with the activation of the enzymes concerned in the building process. The building process often takes place at the expense of complex substances existing in the environment. It is as though we wished to build a laboratory on the site of an apartment; the one structure must be demolished to its constituent units before the new one can be constructed in its place. Consequently the growth of new cells in an environment already utilised by previous growth involves the degradation of the complex proteins, etc., left by the earlier inhabitants, to assimilable material such as amino-acids, ammonia, or even nitrogen, and simple carbon substances. In this chapter we shall be concerned with these breakdown reactions.

Proteolysis

Under this heading we group those reactions involved in the hydrolysis of protein to amino-acids. The series of enzymes involved in such breakdown has been studied with great success in animals, and the proteolytic enzymes of the mammalian intestinal tract have been divided into pepsin, trypsin, "erepsin," polypeptidases, peptidases, etc., but comparatively few studies in detail have been made of the corresponding enzymes formed by bacteria. The native protein molecule is too large to enter the bacterial cell, and consequently if the organism is to utilise such molecules it must first excrete extracellular enzymes to start the hydrolysis. The power to excrete such enzymes in quantity is restricted to comparatively few species. Some of the Clostridia, such as *Cl. histolyticum* and *Cl. sporogenes*, excrete

highly active proteases into their environment. This can be demonstrated by filtering such organisms from culture, when it will be found that the cell-free filtrate contains an active proteolytic enzyme which can be concentrated and precipitated by suitable protein precipitants. The liquefaction of tissues around a wound is due to the proteolytic activities of contaminants of this type. Other genera, such as *Proteus* and *Pseudomonas*, have less marked proteolytic activities, while *Streptococci* are sometimes feebly proteolytic. Even proteolytic organisms will fail to grow when inoculated into a medium containing native protein as sole source of nitrogen, as they require some utilisable source of nitrogen from which to synthesise the extracellular protease necessary to initiate the hydrolysis of the protein.

Once native protein has been hydrolysed to peptones, the majority of the heterotrophic organisms are able to utilise these peptones as sources of nitrogen and/or energy. Thus media in common use in the laboratory for general growth purposes are prepared with a basis of peptone. It is highly probable that genera and species differ widely in the proteolytic enzymes which they produce, but there have not as yet been sufficient studies of this aspect of the subject to make any generalisations possible. The end-products of the breakdown of proteins by bacterial proteases are amino-acids, and discussion of nitrogen metabolism must at present hinge mainly on amino-acid metabolism, as it is here that the widest variety of further breakdown products occur.

AMINO-ACID BREAKDOWN

If we consider the general formula of an amino-acid:

$$R.CHNH_2.COOH$$

we find that there is no theoretically possible mode of attack which is not employed by some organism or other under some condition or other. The molecule can be degraded in three main ways: (1) by removal of the $—NH_2$ group, or deamination; (2) by removal of the —COOH group, or

decarboxylation; or (3) by splitting or hydrolysis of the molecule in some other position. In some cases we find that a single organism may attack an amino-acid by both deamination and decarboxylation, but the two processes do not take place together as the enzymes involved are not formed under the same conditions of growth. When growth takes place in an alkaline medium, the carboxyl group of the amino-acid is ionised, $R.CHNH_2.COO'$, and if the particular amino-acid can be attacked by that organism, the specific deaminase is produced and the organism will attack the amino-acid by removal of the unionised $—NH_2$ group. Conversely, if growth takes place in an acid medium, then the amino-group of the amino-acid is ionised, $R.CHNH_3^+.COOH$, and the specific decarboxylase will be produced so that the organism will attack the amino-acid by removal of the unionised —COOH group. For example, *Esch. coli*, grown in an alkaline medium, will attack L-glutamic acid with the formation of α-keto-glutaric acid and ammonia:

$$HOOC.CH_2.CH_2.CHNH_2.COOH + O \longrightarrow HOOC.CH_2.CH_2.CO.COOH + NH_3,$$

but the same organism grown in an acid medium can no longer attack the molecule by deamination but does so, if at all, by decarboxylation

$$HOOC.CH_2.CH_2.CHNH_2.COOH \longrightarrow HOOC.CH_2.CH_2.CH_2NH_2 + CO_2.$$

The products of amino-acid breakdown may thus be profoundly influenced, not only by the particular organism, but by the *p*H of the medium in which that organism is grown (see Chap. IV).

DEAMINATION

Removal of the $—NH_2$ group from an amino-acid may be accomplished by different bacteria in different ways, such as by oxidation, reduction, desaturation, hydrolysis, etc., of the substrate molecule.

Oxidative deamination

Oxidative removal of the $—NH_2$ group is accomplished according to the equation

$$R.CHNH_2.COOH + O \longrightarrow R.CO.COOH + NH_3,$$

with the production of the α-keto-acid corresponding to the amino-acid attacked. This is the type of breakdown found in mammalian kidney cells, but whereas most amino-acids are attacked by oxidative deamination in these tissues, in bacteria this method of attack is restricted to certain organisms and specific amino-acids. *Esch. coli* is known to deaminate glycine, L-alanine and L-glutamic acid in this way with the formation of glyoxylic, pyruvic, and α-ketoglutaric acids respectively.

$$H_2NCH_2.COOH + O \longrightarrow CHO.COOH + NH_3$$

$$CH_3.CHNH_2.COOH + O \longrightarrow CH_3.CO.COOH + NH_3$$

$$HOOC.CH_2.CH_2.CHNH_2.COOH + O \rightleftharpoons HOOC.CH_2.CH_2.CO.COOH + NH_3.$$

In the case of glutamic acid the reaction is really accomplished in two stages, in which the first stage consists of a dehydrogenation to imino-glutaric acid, which is then spontaneously hydrolysed to ketoglutaric acid.

$$\begin{array}{l} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \quad - 2H \xrightleftharpoons[\text{dehydrogenase}]{\text{Glutamic}} \quad \begin{array}{l} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ C{=}NH \\ | \\ COOH \end{array} \quad + H_2O \rightleftharpoons \quad \begin{array}{l} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ C = O \\ | \\ COOH \end{array} \quad + NH_3$$

This deamination process is essentially similar to that which occurs in mammalian tissues, with the difference that whereas liver L-glutamic acid dehydrogenase requires coenzyme I, the enzyme of *Esch. coli* requires coenzyme II.

Both stages of the reaction are reversible so that L-glutamic acid can be synthesised from ammonia and α-ketoglutaric acid. This may represent the main path of glutamic acid synthesis in bacteria. In mammalian tissues, the glutamic acid dehydrogenase may act as a carrier system for the deamination (or, in reverse, the synthesis) of other amino-acids, as some workers claim that transamination occurs between either α-ketoglutaric acid and any other amino-acid or, alternatively, between glutamic acid and any other α-keto-acid. This may not be the case in bacteria, as so far it has not been possible to demonstrate transamination other than in a very restricted sense in these organisms (see p. 94), and there is definite evidence, on the other hand, that the deamination of some amino-acids passes through reactions quite different from those involved in the postulated glutamic acid carrier system of mammalian tissues.

Esch. coli would seem to attack the three amino-acids mentioned by oxidative deamination, but no others. Stumpf and Green have, however, recently found an L-amino-acid oxidase in *Pr. vulgaris*, *Ps. pyocyanea*, and *Aerobacter aerogenes*, which attacks 11 amino-acids: the *laevo*-isomers of phenylalanine, tyrosine, leucine, *iso*-leucine, methionine, tryptophan, histidine, norleucine, norvaline, amino-butyric acid, and arginine. In each case the corresponding keto-acid is formed according to the equation

$$R.CHNH_2.COOH + O \longrightarrow R.CO.COOH + NH_3.$$

The enzyme has been isolated in a cell-free state by disintegrating a thick suspension of the bacteria with supersonic vibrations. Other common naturally-occurring amino-acids are not attacked in the presence of the enzyme, although the intact organism is capable of a wider range of deaminating activities and must consequently possess other enzymes affecting the deamination of these other amino-acids. The enzyme cannot be obtained from *Esch. coli*, *S. haemolyticus*, *B. subtilis*, or *Staph. aureus*. The suggestion is made that there are several bacterial amino-acid oxidases, and that the specificity of these enzymes varies with their source.

Reductive deamination

In this case hydrogen is added to the substrate with the production of a saturated fatty acid:

$$R.CHNH_2.COOH + 2H \longrightarrow R.CH_2.COOH + NH_3.$$

This type of deamination has been demonstrated with certain strict aerobes (e.g. *Mycob. phlei*) in the case of aspartic acid, which is reduced to succinic acid with the liberation of ammonia:

$$HOOC.CH_2.CHNH_2.COOH + 2H \longrightarrow HOOC.CH_2.CH_2.COOH + NH_3.$$

Desaturation deamination

In this case NH_3 is removed from the amino-acid molecule, leaving an unsaturated fatty acid:

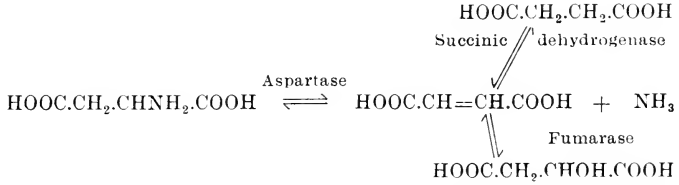
$$R.CH_2.CHNH_2.COOH \longrightarrow R.CH = CH.COOH + NH_3.$$

When intact cells of *Esch. coli* deaminate aspartic acid, the final product is succinic acid, but if the deamination takes place in the presence of certain inhibitors such as toluene, then the end-product is not succinic acid but fumaric acid, and the deamination takes place according to the equation:

$$HOOC.CH_2.CHNH_2.COOH \longrightarrow HOOC.CH = CH.COOH + NH_3.$$

In the absence of inhibitors fumaric acid is reduced to succinic acid. The enzyme responsible for the desaturation deamination is called "aspartase," and has been isolated in a cell-free state. The aspartase reaction is reversible so that aspartic acid can be synthesised from ammonia and fumaric acid. Since the reaction is reversible, the end-products of either forward or back reactions form an equilibrium mixture of ammonia, fumaric acid, and aspartic acid. When the intact organism is used as source of the enzyme, the equilibrium mixture is further complicated by the presence of another reversible enzyme, fumarase, which catalyses the hydration of fumaric acid to malic acid. In the intact organism the deamination of aspartic acid may lead to the formation of

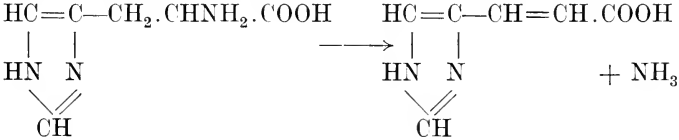
any or all of the following products: ammonia, fumaric acid, succinic acid, malic acid.



Aspartase is found in many facultative anaerobes but is not, apparently, involved in the reductive deamination of aspartic acid to succinic acid by certain strict aerobes (as above).

When cell preparations of aspartase are left to stand on the bench, especially if the *p*H is adjusted to around 4·0, the activity steadily declines. The lost activity can be restored if biotin is added to the cell suspension, which suggests that a biotin-containing coenzyme is involved in aspartase action. However, adenylic acid can also restore the lost activity but much larger concentrations are required. The cell-free aspartase has now been resolved into specific protein and coenzyme portions, and the protein can be activated by the addition of both adenylic acid and biotin, neither being active alone. It is probable that adenylic acid and biotin combine, in the presence of an enzyme contained in the preparation, to form a complex active as co-aspartase.

A further example of desaturation deamination is the breakdown of histidine by *Esch. coli* to give urocanic acid, so called since it was first isolated from the urine of dogs.



Hydrolytic deamination

A method of deamination that is theoretically possible involves hydrolysis to the corresponding hydroxy-acid:

$$R.CHNH_2.COOH + H_2O \longrightarrow R.CHOH.COOH + NH_3$$

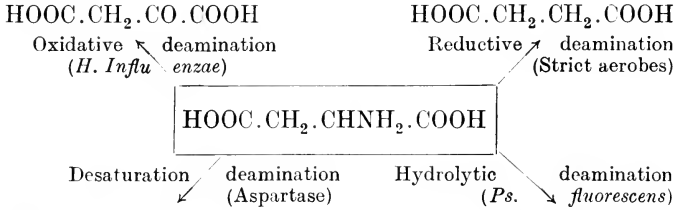
but, with one exception, such a reaction has not been demonstrated in bacteria. A claim has been made that aspartic acid is hydrolytically deaminated to malic acid by *Ps. fluorescens liquefaciens*:

$$HOOC.CH_2.CHNH_2.COOH + H_2O \longrightarrow HOOC.CH_2.CHOH.COOH + NH_3.$$

The evidence for the reaction is not direct and requires confirmation with a cell-free enzyme system.

Aspartic acid

Since aspartic acid is oxidatively deaminated to oxalacetic acid by *Haemophilus influenzae*, it provides an example of a substrate which can be deaminated in the four ways so far discussed:



Dehydration deamination

There is one particular example of this type of deamination and that is the breakdown of L-serine by *Esch. coli*; the postulated course of the breakdown is as follows:—

$$\begin{array}{l} CH_2OH \\ | \\ CHNH_2 \\ | \\ COOH \end{array} - H_2O \longrightarrow \begin{array}{l} CH_2 \\ \| \\ C{-}NH_2 \\ | \\ COOH \end{array} \rightleftharpoons \begin{array}{l} CH_3 \\ | \\ C{=}NH \\ | \\ COOH \end{array} + H_2O \rightleftharpoons \begin{array}{l} CH_3 \\ | \\ C{=}O \\ | \\ COOH \end{array} + NH_3$$

The experimental fact is that serine is attacked anaerobically to liberate pyruvic acid and ammonia. To explain this reaction the above steps have been postulated, starting with a dehydration of serine to the unsaturated amino-acid by an enzyme called "serine dehydrase." Preparations of washed

cells lose their serine dehydrase activity under conditions similar to those described for loss of aspartase activity and can also be restored by the addition of biotin. This type of deamination is only made possible by the unique structure of the serine molecule. An analogous reaction occurs with cysteine, when the first step is a removal of H_2S by "cysteine desulphurase," after which the course of the breakdown is presumably the same as that postulated for serine:

$$\begin{array}{l} CH_2SH \\ | \\ CHNH_2 \\ | \\ COOH \end{array} - H_2S \longrightarrow \begin{array}{l} CH_2 \\ \| \\ C{-}NH_2 \\ | \\ COOH \end{array} \rightleftharpoons \begin{array}{l} CH_3 \\ | \\ C{=}NH \\ | \\ COOH \end{array} + H_2O \rightleftharpoons \begin{array}{l} CH_3 \\ | \\ C{=}O \\ | \\ COOH \end{array} + NH_3$$

The cysteine enzyme has also been obtained in a cell-free condition from *Esch. coli*; it is inactivated by dialysis and the activity restored by the addition of zinc, magnesium, or manganese.

Deamination by the strict anaerobes

Some of the Clostridia employ specific methods for the deamination of some amino-acids. *Cl. sporogenes* was first studied by Stickland, who found that washed suspensions of this organism are unable to deaminate any amino-acid if this is added by itself to the suspension. Using reducible dyes as H-donators and acceptors, he found that some amino-acids are deaminated in the presence of a H-acceptor and some in the presence of a H-donator dye; in other words some amino-acids act as H-donators and some as H-acceptors. If two amino-acids, one from each group, are added together to the suspension of organisms, then deamination of both occurs according to the general equation:

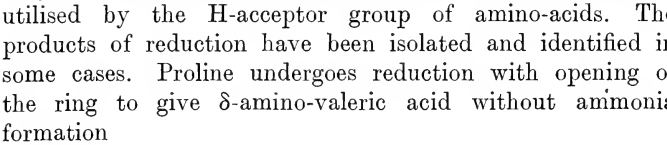
$$\begin{array}{l} R \\ | \\ CHNH_2 \\ | \\ COOH \end{array} + H_2O + \begin{array}{l} X \\ | \\ CHNH_2 \\ | \\ COOH \end{array} \longrightarrow \begin{array}{l} R \\ | \\ CO \\ | \\ COOH \end{array} + 2NH_3 + \begin{array}{l} X \\ | \\ CH_2 \\ | \\ COOH \end{array}$$

In this reaction the molecule $R.CHNH_2.COOH$ undergoes

oxidative deamination, the H-acceptor being another amino-acid molecule rather than a coenzyme as in the deamination of glutamic acid by *Esch. coli*. It is possible that a coenzyme is involved as carrier in the "Stickland" reaction, but the enzyme kinetics of the reaction have not yet been studied in detail. The amino-acids so far tested fall into the following groups:—

H-acceptors	*H-donators*	
Glycine	Alanine	Leucine
Proline	Valine	Phenylalanine
Hydroxyproline	Cysteine	Serine
Ornithine	Histidine	Aspartic acid
Arginine	Glutamic acid	
Tryptophan		

Cl. sporogenes also possesses a very active hydrogenase enzyme activating molecular hydrogen so that it can be utilised by the H-acceptor group of amino-acids. The products of reduction have been isolated and identified in some cases. Proline undergoes reduction with opening of the ring to give δ-amino-valeric acid without ammonia formation



Ornithine, on the other hand, is also reduced to δ-amino-valeric acid but with the liberation of one molecule of ammonia,

$$CH_2NH_2.CH_2.CH_2.CHNH_2.COOH + 2H \longrightarrow CH_2NH_2.CH_2.CH_2.CH_2.COOH + NH_3.$$

Glycine is reductively deaminated to acetic acid, and the Stickland reaction between glycine and alanine takes place as follows, presumably with the intermediate formation of pyruvic acid from alanine,

$$2CH_2NH_2.COOH + CH_3.CHNH_2.COOH + 2H_2O = 3CH_3.COOH + 3NH_3 + CO_2.$$

This oxido-reduction reaction between two amino-acids, usually called the "Stickland reaction," would seem to be specific for certain Clostridia such as *Cl. sporogenes* and *Cl. botulinum*, but is not carried out by all Clostridia. Other members of the genus employ a different method of deamination in which single amino-acids are attacked with the liberation of ammonia and gaseous hydrogen. The growth of certain Clostridia on meat media results in the formation of considerable volumes of gas, hence the name "gas gangrene" given to the clinical condition following the infection of wounds with certain pathogenic Clostridia. The greater part of this gas is liberated during the deamination of certain amino-acids. For example, *Cl. tetanomorphum* attacks tyrosine and histidine with the liberation of hydrogen and ammonia. In most cases the products of the deamination have not been fully identified and we do not know how the hydrogen is formed during the deamination process, but it has been suggested that we have here a form of oxidative deamination, consisting of dehydrogenation followed by release of the hydrogen as molecular hydrogen instead of combination with a H-acceptor.

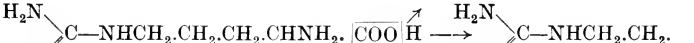
DECARBOXYLATION

The removal of the terminal —COOH group of an amino-acid is carried out by specific amino-acid decarboxylases with the formation of the corresponding amine:

$$R.CHNH_2.COOH \longrightarrow R.CH_2NH_2 + CO_2.$$

The decarboxylases are specific for the natural isomer of one amino-acid. From studies of these enzymes in a cell-free state, it seems that only such amino-acids are attacked as have at least one chemically active (polar) group in the molecule other than the terminal —COOH and the α-NH_2 groups. Thus decarboxylases have been described for arginine, lysine, ornithine, histidine, tyrosine, glutamic acid,

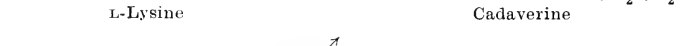
aspartic acid, and possibly tryptophan, but for no monamino-monocarboxylic acids. In each case a simple decarboxylation occurs with the production of the corresponding amine or, with the dicarboxylic acids, of the ω-amino-acid. Any alteration in the structure of the amino-acid such as methylation of either $—NH_2$ group in the diamino-acids, or of substitution of the —OH in tyrosine, or the δ-COOH in glutamic acid, results in inactivation, since combination between the decarboxylase protein and the substrate is thus prevented (see Chap. III). The addition of —OH to the substrate such as occurs in hydroxylysine, dihydroxytyrosine, or hydroxyglutamic acid slows down, but does not prevent, decarboxylation by the corresponding decarboxylase.



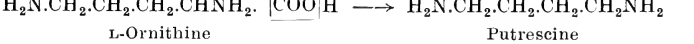
L-Arginine — Agmatine

L-Lysine — Cadaverine

L-Ornithine — Putrescine



L-Histidine — Histamine



L-Tyrosine — Tyramine

L-Glutamic acid — γ-Amino-butyric acid

L-Aspartic acid — β-alanine

Many of the biologically produced amines have physiological or pharmacological activities, and so their production *in vivo* by bacteria might have important consequences. For example, histamine, produced by the decarboxylation of histidine, is known as a "depressor substance" in that injection of small quantities into an animal results in a rapid fall in blood pressure. It also produces contraction of smooth muscle and causes a general condition in the animal analogous to "shock." Tyramine, on the other hand, is a "pressor substance" in that injection causes a rise of blood pressure. Its general properties are the opposite of those of histamine, and its action on injection is similar to that of adrenaline, but much less active weight for weight. Since the action of tyramine resembles that of adrenaline which is secreted by sympathetic nerve endings, tyramine is said to be a "sympatheticomimetic" drug, while histamine is "parasympatheticomimetic." The other amines are less active and, in general, the diamines such as putrescine and cadaverine (from ornithine and lysine respectively) have weak parasympatheticomimetic activities, while the mon-amines have weak sympatheticomimetic activities. The guanidine nucleus in agmatine, produced by decarboxylation of arginine, confers an insulin-like activity upon this amine but it is not possible to use it as an insulin substitute as its repeated administration gives rise to liver damage. The products of decarboxylation of the dicarboxylic acids have no known pharmacological properties, although β-alanine is a growth factor for some micro-organisms and forms part of the pantothenic acid molecule.

The amino-acid decarboxylases are formed only when growth takes place in an acid environment, and they have unusually acid activity-*p*H optima varying from 2·5 for histidine decarboxylase (*Cl. welchii*) to 5·5 for the ornithine decarboxylase of *Cl. septicum*. Six of the enzymes have been obtained in a cell-free condition and five of them, the decarboxylases of lysine, arginine, ornithine, tyrosine, and glutamic acid, have pyridoxal phosphate as prosthetic group. The histidine decarboxylase apparently does not require this prosthetic group.

The distribution of the amino-acid decarboxylases amongst bacteria appears to be haphazard (see Table XIV). *Esch. coli* may have the decarboxylases specific for arginine, lysine, ornithine, histidine, glutamic acid, and very occasionally tyrosine, but wide strain variations are found as some strains may have five of these enzymes while others may have two, one, or none. Many Streptococci possess tyrosine decarboxylase, but strains differ widely in the activity of the enzyme. Clostridia again show wide differences; *Cl. welchii* may possess both histidine and glutamic acid decarboxylases, *Cl. septicum* ornithine decarboxylase only, and many other species have no decarboxylases. Aspartic acid decarboxylase has been found in the symbiotic nitrogen-fixing organisms *Rhizobia*.

TABLE XIV

DISTRIBUTION OF AMINO-ACID DECARBOXYLASES

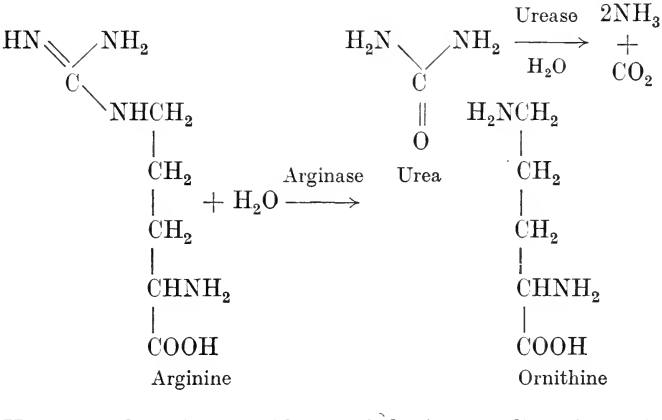
Organism	Decarboxylase Substrate:						
	Arginine	Histidine	Lysine	Tyrosine	Ornithine	Glutamic acid	Aspartic acid
Esch. coli	+	+	+	+	+	+	−
S. haemolyticus ...	−	−	−	+	−	−	−
Proteus vulgaris ...	−	−	−	−	+	+	−
Cl. welchii	−	+	−	−	−	+	−
Cl. septicum ...	−	−	−	−	+	−	−
Cl. aerofoetidum ...	−	−	−	+	−	+	−
Cl. sporogenes ...	−	−	−	−	−	−	−
Rhizobium leguminosarum	−	−	−	−	−	−	+

SPLITTING OF THE MOLECULE

Two examples of this type of amino-acid degradation will be discussed.

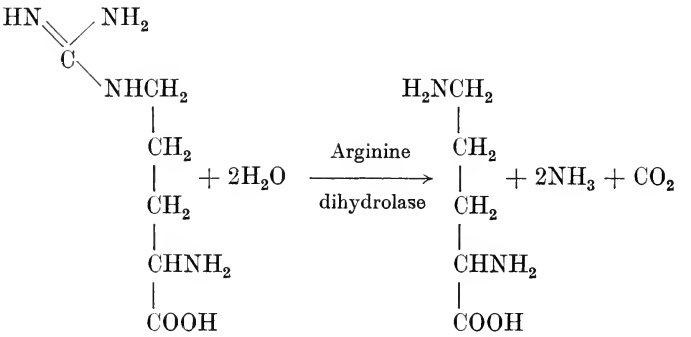
Arginine dihydrolase

The Gram-positive Streptococci and Staphylococci are very exacting in their amino-acid requirements, and this synthetic disability is accompanied by very restricted catabolic activities. The only amino-acid from which these organisms can liberate ammonia rapidly is arginine. The breakdown of arginine is not a simple decarboxylation or deamination, as analysis of the products shows that these are ammonia, carbon dioxide, and ornithine. In mammalian liver we get a somewhat similar breakdown of arginine in which urea is first split from arginine by arginase, and can then be decomposed to ammonia and carbon dioxide by urease:



However, there is no evidence of the intermediate formation of urea during the degradation of arginine by Streptococci, and these organisms do not possess urease. The reaction is presumably a direct hydrolysis as shown below, and the enzyme

concerned has been called "arginine dihydrolase" to distinguish it from the arginase of liver cells.



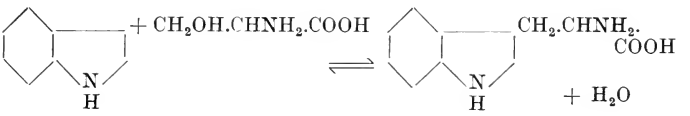
Arginine dihydrolase is possessed by most Streptococci to varying extent. Its function is not clear, as arginine is an essential amino-acid for the organisms which consequently appear to attack one of their essential nutrients. It is possible that these organisms, which carry out a simple *homo*lactic fermentation of glucose, depend upon arginine dihydrolase action for the provision of the carbon dioxide which is essential for their growth.

The production of indole from tryptophan

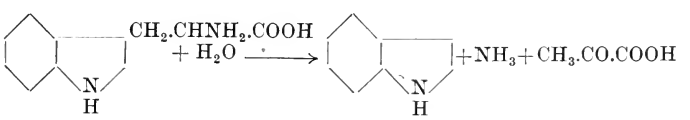
In Chap. II various biochemical tests were outlined for the systematic characterisation of bacteria. One of these tests is the formation of indole in protein-containing and protein-digest media by *Esch. coli*. It has been known from the early days of bacteriology that some organisms, particularly those of the *coli* group, produce a strongly smelling substance from protein digests, and that this substance reacts with *p*-dimethyl-amino-benzaldehyde in alcoholic HCl to produce a pink colour. The substance was known as indole, and Ehrlich worked out a simple application of the colour reaction for testing the production of indole in cultures. In 1901 Hopkins and Cole showed that the precursor of

indole is tryptophan. The bacterial reaction occurs aerobically, and the oxidation of one molecule of tryptophan to indole is accompanied by the consumption of five atoms of oxygen. We can make guesses at the nature of possible intermediate substances, but if theoretically possible intermediates such as indole-acetic, -propionic, -pyruvic, -acrylic, -lactic acids, or the corresponding aldehydes, are added either to cultures or to washed suspensions of active organism, they do not give rise to indole and consequently cannot be indole precursors. In an atmosphere of hydrogen *Esch. coli* produces indole-propionic acid from tryptophan, but this must involve a different metabolic path from that followed aerobically, as indole-propionic acid is not decomposed further under aerobic conditions. It seems that the first step in the aerobic decomposition of tryptophan is not related to those steps discovered for other amino-acids.

Recent studies with the bread mould, *Neurospora crassa*, have shown that tryptophan is synthesised by this organism from indole and L-serine and that the reaction is reversible:



The same workers suggested that a similar reaction is involved in the synthesis and breakdown of tryptophan by *Esch. coli*. Further studies have not confirmed this suggestion, as a cell-free preparation has been obtained from *Esch. coli*. and the breakdown of tryptophan by the preparation results in the liberation of indole, ammonia, and pyruvic acid.



The preparation will not attack either serine or alanine so these cannot be intermediates in the reaction. The oxygen

TABLE XV

AMINO-ACID METABOLISM OF BACTERIA

Amino-acid	*Esch. coli*	*Streptococci*	*Cl. sporogenes*	*Cl. welchii*
Glycine	Glyoxalate + NH_3	—	(H-acceptor) Acetate + NH_3	—
L-Alanine	Pyruvate + NH_3	—	(H-donator) Pyruvate + NH_3	—
L-Glutamate	Ketoglutarate + NH_3 δ-Amino-butyrate + CO_2	—	(H-donator)	— δ-Amino-butyrate + CO_2
L-Aspartate	Fumarate + NH_3	—	(H-donator)	—
DL-Serine	Pyruvate + NH_3	—	(H-donator)	? + H_2 + NH_3
L-Cysteine	Pyruvate + H_2S + NH_3	—	(H-donator)	? + H_2S + H_2 + NH_3
L-Tyrosine	Phenol + NH_3 Tyramine + CO_2	— Tyramine + CO_2		—
L-Tryptophan	Indole + NH_3 + $CH_3.CO.COOH$ Indole-propionate + NH_3 (anaer)	—	(H-acceptor) Indole-propionate + NH_3	—
L-Histidine	Urocanate + NH_3 Histamine + CO_2	—	(H-donator)	— Histamine + CO_2
L-Arginine	? + NH_3 Agmatine + CO_2	Ornithine + NH_3 + CO_2	(H-acceptor) δ-Amino-valerate + NH_3	? + NH_3 + CO_2
L-Ornithine	— Putrescine + CO_2	—	(H-acceptor) δ-Amino-valerate + NH_3	—
L-Lysine	Cadaverine + CO_2	—	—	—
L-Proline	?	—	(H-acceptor) δ-Amino-valerate + NH_3	—

— = not attacked. ? = product not identified.

consumption accompanying indole formation must be involved in the oxidation of pyruvic acid.

The amino-acid metabolism of *Esch. coli*, Streptococci, and two typical Clostridia is summarised in Table XV.

FOR FURTHER READING

"Enzymes Involved in the Primary Utilisation of Amino-acids by Bacteria," Gale, E. F., *Bact. Rev.*, 1940, **4**, 135.

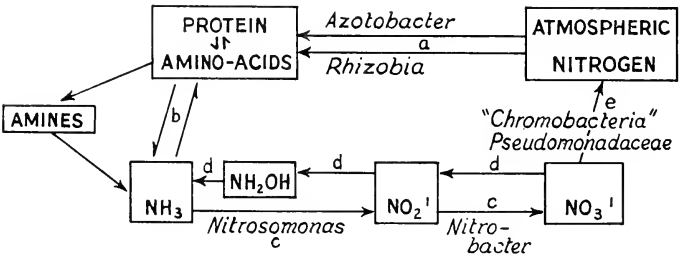
"Bacterial Amino-acid Decarboxylases," Gale, E. F., *Advances in Enzymology*, 1946, **6**, 1.

"Nitrogen Metabolism," Gale, E. F., *Annual Rev. Microbiol*, 1947, **1**, 141.

CHAPTER X

THE NITROGEN CYCLE

Gaseous nitrogen is fixed by certain bacteria with the formation of organic nitrogenous matter. This organic material is decomposed by other organisms with the production of ammonia. Ammonia is oxidised to nitrate by the nitrifying autotrophes, and certain Chromobacteria have the property of liberating gaseous nitrogen from nitrate. So nitrogen completes a cycle: the steps in this cycle are indicated below and will now be considered in greater detail.



(*a*) Nitrogen-fixation

In Chap. V it was shown that certain organisms are able to utilise atmospheric nitrogen as a source of nitrogen for growth purposes. This fact has been used since very early days of agriculture as a means of fertilising soil. The majority of plants lead an autotrophic type of existence and draw upon the inorganic nitrogen of the soil for their nitrogen requirement. Consequently the growth of a heavy crop of grain results in the depletion of the soil-nitrogen, and cropping of the same soil year after year results in a steadily decreasing yield of grain until eventually such cultivation is no longer economical. Since the times of Virgil it has been known that this depletion can be countered in one of two ways:

either by leaving the soil fallow for a year or, alternatively, by growing a crop of clover, vetches, alfalfa, or other leguminous plant. Either of these measures results in a replenishment of the soil-nitrogen, and it is possible to grow further successful crops of grain. Both of these natural fertilisation measures owe their efficacy to the action of bacteria in "fixing" atmospheric nitrogen and so rendering it available in the soil in a form which can be utilised by plants.

In the case of the field left fallow, the organism mainly concerned is the strict aerobe, *Azotobacter*. Berthelot showed in 1885 that if soil is left exposed to the air, then its nitrogen content slowly increases and that this increase takes place at the expense of atmospheric nitrogen. He further showed that the responsible agent is biological, since the process can be stopped by heat or by treating the soil with caustic chemicals. It was some years before any organism was isolated from soil which has the property of fixing nitrogen and the first such organism isolated was *Cl. pastorianum*, a strict anaerobe which is of less importance than *Azotobacter*. *Azotobacter* is able to grow rapidly in the presence of gaseous nitrogen as sole source of nitrogen and of carbohydrate as carbon and energy source. Growth is such that there is a constant ratio between nitrogen fixed and carbohydrate utilised. In soil the limiting factor is often the amount of carbohydrate available, and this explains the practice of some Indian farmers of enriching their soil by ploughing waste molasses into it. Despite many studies on *Azotobacter*, we are still without any definite knowledge of the chemistry of the fixation process (see p. 90).

In the days before artificial fertilisers were available, the alternative open to the farmer, instead of leaving his fields fallow, was to cultivate on his fields a crop of one of the leguminous plants, *i.e.* those plants having nodules on their roots. The function of the root-nodules was first made apparent in 1888 by Hellriegel and Wilfarth, who studied the growth of peas and the formation of root-nodules on their

roots in soil and sand under controlled conditions. Their results may be summarised as follows:—

Condition of Soil	Presence of Fixed-nitrogen	Growth	Formation of Root-nodules
Normal ...	+/−	+++	+
Sterile ...	−	−	−
Sterile ...	+	++	−
Non-sterile ...	−	+++	+

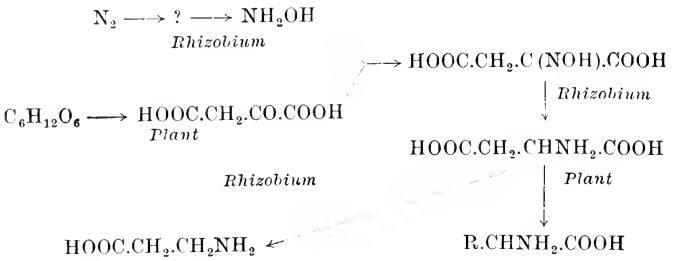
In normal soil, normal growth occurs and nodules are formed on the roots; analysis shows that an increase in the nitrogen-content of the system (soil + plant) has occurred during growth. If the peas are sown into sterile sand from which all fixed-nitrogen has been washed away, then no growth can occur and no nitrogen-fixation takes place. If the sterile washed sand is treated with fixed-nitrogen in the form of nitrate, etc., then growth occurs, but no formation of nodules takes place, and analysis shows that the nitrogen-content of the system (soil + plant) has remained constant, so that nitrogen-fixation has not occurred. On the other hand, if the soil has been freed from fixed-nitrogen but not sterilised, then normal growth takes place, nodules appear on the roots, and the entire nitrogen-content of the plant is obtained by fixation of atmospheric nitrogen. These experiments demonstrate that (1) nitrogen-fixation occurs only in the presence of root-nodules, and (2) nodules never form on the roots if growth takes place in sterile soil.

The formation of nodules on the roots of these plants is due to the action of bacteria called *Rhizobia*, which exist in soil and are able to penetrate the root-hairs of certain plants with which they come into contact. When penetration has taken place the root-cells in the vicinity of the invading bacteria are stimulated to division and the increased growth so produced gives rise to the nodule. The *Rhizobia* in contact with the plant within the nodule are now able to carry out

nitrogen-fixation, but the process is essentially a symbiotic one, in that neither the plant alone nor the free-living *Rhizobium* can fix nitrogen. The plant, as shown above, can grow in sterile soil as long as it is provided with a source of fixed-nitrogen, but it cannot utilise atmospheric nitrogen under these conditions. *Rhizobium* can lead a free-living existence and can grow normally in a medium containing fixed-nitrogen, but it cannot fix atmospheric nitrogen when growing apart from a host-plant. There is an important specificity between the particular *Rhizobium* and the plant with which it can enter into a symbiotic relation, thus *Rh. trifolium* can form nodules only on the roots of clover, and can fix nitrogen only in symbiosis with that plant, while *Rh. leguminosarum* can form nodules only on the roots of the pea. Further than this, some strains of *Rh. trifolium*, for instance, are better nitrogen-fixers in symbiosis with clover than are others, and it often pays a farmer nowadays to obtain a suitable strain with which to inoculate his soil before sowing this type of crop.

The chemistry of the symbiotic nitrogen-fixation process has been the subject of much work in Helsinki University and also in American laboratories. Prof. Virtanen of Helsinki has published a series of papers in which he claims to have elucidated the chemical processes involved but, unfortunately, attempts to confirm this work in other laboratories have not, so far, met with success. The scheme put forward by Virtanen was as follows:—

Nitrogen is fixed by *Rhizobia* with the formation, after unknown intermediate stages, of hydroxylamine. At the same time carbohydrate is broken down within the host-plant with the formation of oxalacetic acid. Hydroxylamine and oxalacetic acid combine spontaneously to form the corresponding oximo-succinic acid which is then reduced by the organism to aspartic acid. The aspartic acid, in turn, acts as a source of other amino-acids in the plant through transamination.



The evidence put forward by Virtanen in support of this scheme includes the following points:—

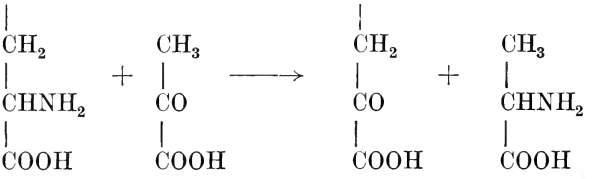
1. The demonstration that aspartic acid and its decarboxylation product, β-alanine, are excreted into the soil around the roots of young pea plants, and that this excretion occurs only from the nodules.

2. The demonstration of oxalacetic acid in the sap of the host-plant.

3. The isolation of oximo-succinic acid from the soil around the nodules.

4. The demonstration that *Rhizobia* can grow on a nitrogen-free medium provided that oxalacetate is added to the medium —when growth occurs with nitrogen-fixation.

5. That transamination between aspartic acid and pyruvic acid will take place in the presence of extract of pea with the formation of oxalacetic acid and alanine.



This evidence is quite formidable, and would seem to provide satisfactory confirmation of the fixation hypothesis, but

several key-points, namely the proof of aspartic acid excretion by the nodules and of the growth of *Rhizobia* in nitrogen-free oxalacetate media, have not so far been confirmed in other laboratories. Also the evidence of the nature of the oximo-succinic acid isolated from soil is not altogether convincing. Wilson, in America, has carried out much careful and detailed research into the symbiotic nitrogen fixation process, and has not obtained evidence in confirmation of Virtanen's hypothesis. An interesting point discovered by this group of workers is that the fixation process is inhibited by the presence of hydrogen.

The root-nodules contain a haematin-pigment resembling haemoglobin, but the part played by this substance in the fixation process has not yet been elucidated.

(*b*) Interchange of bacterial nitrogen and ammonia

The interchanges between bacterial protein, amino-acids, and ammonia have been dealt with in detail in these last two chapters. Degradation of amino-acids will take place either by deamination or by decarboxylation, according to the *p*H holding in the environment at the time. If decarboxylation occurs, the nitrogenous product is an amine, and if the environmental *p*H subsequently returns to neutral or alkaline values, then amines themselves are oxidised by certain bacteria, particularly *Pseudomonas*, with the liberation of ammonia.

(*c*) Nitrification

The oxidation of ammonia to nitrate in the soil is called "nitrification," and the biological nature of the process was first demonstrated in 1877 by Schloesing and Muntz. They poured sewage effluent through a long tube containing sand and chalk, and showed that, after the fluid had been passing through the tube for a few days, ammonia entering the tube was removed during the passage of the effluent through the tube, and replaced by nitrate in the fluid issuing from the tube. They showed that treatment of the contents of the tube with

heat or caustic chemicals stopped the nitrification process so that ammonia passed through unchanged. If the passage of the sewage effluent were maintained, then the culture was slowly re-established and nitrification resumed after a few days. It was thirteen years after this demonstration that the first nitrifying organism was isolated in pure culture. In Chap. V the nitrifying organisms *Nitrosomonas* and *Nitrobacter* are described as strict autotrophes whose growth is inhibited by the presence of organic matter. Since it was the custom in those days to attempt the isolation of organisms by growth on the surface of solidified gelatine, it is understandable why no successful isolation of nitrifying bacteria was achieved for some time. It was not until Winogradsky invented the purely inorganic solid medium consisting of inorganic salts incorporated in a silicic acid gel that the first successful isolation of a nitrifier was accomplished. It then became obvious that two organisms are involved in the nitrifying process, the first (*Nitrosomonas*) carrying out oxidation of ammonia to nitrite, and the second (*Nitrobacter*) completing the oxidation of nitrite to nitrate. The two processes are not interchangeable, for while *Nitrosomonas* can grow on a synthetic medium containing ammonia as source of nitrogen and energy,

$$2NH_3 + 3O_2 \longrightarrow 2HNO_2 + 2H_2O + 79 \text{ Cals.}$$

it cannot grow on a medium containing nitrite but no ammonia. Similarly, *Nitrobacter* cannot grow in a nitrite-free medium, but must obtain its energy from the oxidation of nitrite to nitrate:

$$HNO_2 + O \longrightarrow HNO_3 + 21{\cdot}6 \text{ Cals.}$$

The process of nitrification is peculiar to these two organisms, and can consequently only take place when conditions are suitable for the growth of strictly autotrophic organisms.

(*d*) Reduction of nitrate to ammonia

This change is one that can be accomplished by several organisms, including *Esch. coli* and *Cl. welchii*. In the presence of a hydrogen donator, *Esch. coli* can reduce nitrate to nitrite

by means of an enzyme called " nitratase." Hydrogen may be supplied by a dehydrogenase such as, for example, formic dehydrogenase, so that the organisms can oxidise formic acid anaerobically in the presence of nitrate:

$$H.COOH + HNO_3 \longrightarrow CO_2 + H_2O + HNO_2.$$

Both *Esch. coli* and *Cl. welchii* possess an active hydrogenase (p. 48) and in the presence of hydrogen, we find that the reduction of nitrate proceeds further than nitrite on to ammonia, with the probable intermediate formation of hydroxylamine:

$$H_2 \rightleftharpoons 2H$$

$$2H + HNO_3 \longrightarrow HNO_2 + H_2O$$

$$4H + HNO_2 \longrightarrow NH_2OH + H_2O$$

$$2H + NH_2OH \longrightarrow NH_3 + H_2O$$

or, the over-all reaction:

$$HNO_3 + 4H_2 \longrightarrow NH_3 + 3H_2O.$$

The interchange between ammonia and nitrate is thus reversible, but whereas the forward reaction (*c*) can be carried out by certain strict autotrophes only, the reduction process (*d*) can be performed by a number of heterotrophic organisms, both strictly and facultatively anaerobic.

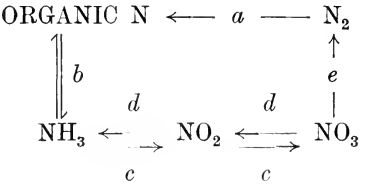
(*e*) Denitrification

When certain species of *Serratia*, Chromobacteria, and *Pseudomonadaceae* are grown in media containing either nitrate or nitrite as source of nitrogen, there is a disappearance of fixed-nitrogen from the culture and bubbles of gaseous nitrogen form in the culture fluid. The chemistry of this denitrification process has not yet been worked out; it is not reversible. The " Chromobacteria " thus provide the final link in the cycle which starts with the fixation of atmospheric nitrogen by *Azotobacter*, etc., passes through the heterotrophic interchange of organic nitrogenous compounds with final degradation to ammonia, through autotrophic

nitrification, and finally to denitrification and the liberation of gaseous nitrogen again.

Conditions affecting the nitrogen cycle

Whenever we have a thoroughly mixed bacterial population, we have the possibility that organisms and enzymes for the entire nitrogen cycle will be present. Which particular part of the cycle will predominate at any moment will depend on conditions holding in the immediate environment. The various reactions in the cycle will be conditioned as follows:—



Reaction (*a*) can occur only in the absence of fixed-nitrogen.

(*b*) Breakdown to ammonia will occur as long as there is excess of organic-nitrogen; if ammonia is the more abundant and a source of carbon is present, then organic-nitrogen will be synthesised at the expense of ammonia.

(*c*) Cannot occur in the presence of organic matter.

(*d*) Can occur only in the presence of organic matter or hydrogen as H-donator.

(*e*) Occurs in presence of nitrate or nitrite as sole source of nitrogen.

Sewage purification

The nitrogen cycle can function in whole or in part in the soil and is put to use in sewage purification. Raw sewage as it comes to the sewage farm contains much organic material and is heavily inoculated with heterotrophic organisms. In the settling tanks proteolytic organisms digest the solid material forming utilisable organic-nitrogen, which is broken down by heterotrophic action to ammonia. The fluid sewage

is then usually treated to some aeration process or trickled over coke filter beds in which a high degree of aeration takes place. In the upper layers of the beds the breakdown of organic-nitrogen is completed and organic matter extensively oxidised. In the lower layers conditions are suitable for the growth of autotrophes and nitrification takes place. The bacterial population of the filter bed is very mixed and a certain amount of denitrification may also take place, the composition of the final effluent depending upon the design of the bed, the speed of flow of sewage, the time since the bed was rested, etc. If the filter bed is kept in continual use the coke becomes " choked " with bacterial growth and the purification process slows down. Consequently the beds have to be rested periodically by stopping the flow of sewage for a few weeks, when the filters clear themselves by auto-digestion, during which the cycle is repeated on a smaller scale, having bacterial protein as starting point instead of sewage.

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The Biochemistry of the Symbiotic Nitrogen Fixation Process, Wilson, P. W. (Univ. Wisconsin Press, Madison).

CHAPTER XI

PATHOGENICITY; CHEMOTHERAPY

From a medical point of view, bacteria are divided into "pathogens" or organisms capable of causing disease in a host, and "saprophytes" or organisms which are harmless to other creatures. Those organisms, such as the normal bacterial flora of skin, mouth, and intestine, which live in constant association with man without causing any disease or lesion, are called "commensals." An organism may be potentially pathogenic in one situation and a commensal in another as, for example, *Cl. welchii* which is a normal harmless inhabitant of the intestine, but is pathogenic should it get into a deep wound.

The majority of bacteria are saprophytic, and in this chapter we intend to consider, as briefly as possible and from a biochemical aspect, what it is that differentiates a pathogenic from a saprophytic organism.

The healthy interior tissues of animals are sterile, and are maintained so by the action of both fixed and wandering cells which have the power to ingest and digest bacteria by the process known as phagocytosis. In mammalian blood, for example, certain of the white corpuscles have this phagocytic property, and if a foreign particle or organism enters the blood-stream, then these phagocytes are attracted towards the foreign substance, surround it, and attempt to destroy or engulf it. Other phagocytic cells are fixed to the capillary walls of the liver, etc., and the whole complex of fixed and wandering scavenging cells is known as the "reticulo-endothelial system." When an organism enters the blood-stream or other tissue, a race ensues between the capacity of the organism to multiply on the one hand, and the capacity of the phagocytes to destroy the invading cells on the other. If the phagocytes win rapidly no disease symptoms appear, but if the bacteria are able to paralyse or outgrow the phagocytes, then there usually follows some disturbance of

the organisation or metabolism of the host, which becomes apparent as clinical disease.

Bacteria may enter the tissues in a variety of ways: through the respiratory passages, through the tonsils, through the intestinal wall or, above all, through any type of wound. If saprophytic bacteria enter by any of these routes, they are rapidly and effectively destroyed by the reticulo-endothelial system, but a pathogenic organism is able to grow within the host's tissues and its capacity to do so is a measure of its

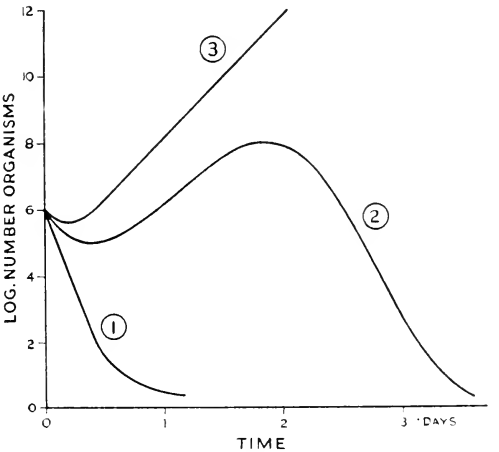


Fig. 13.

" virulence." Fig. 13 shows the effect of injecting 1 million streptococci into the blood-stream of a healthy animal. If the organisms belong to an avirulent strain (Case 1), then there is a steady decrease in the number of bacterial cells in the blood-stream from the time of injection until eventual disinfection. If the strain is highly virulent (Case 3), then, after an initial decrease in numbers, the organism begins to multiply rapidly and there is a steadily increasing number of organisms in the blood-stream until eventually the host dies as a result of their activities. In the intermediate case of a

moderately virulent organism (Case 2), there is the usual small decrease, followed by increasing numbers—during which phase clinical symptoms appear—then a period during which the organisms are being countered by the defence mechanisms of the host, and finally a clearing of the blood-stream as phagocytosis is successful.

Virulent organisms differ from avirulent forms in two main respects. In the first place, virulent bacteria often possess a capsule which protects them against the action of the phagocytes. Virulent pneumococci possess a capsule composed of polysaccharide and if this capsule is removed by enzymatic means, the organism, though viable, is no longer virulent and is rapidly removed *in vivo* by the cells of the reticulo-endothelial system. The protective capsule is not always polysaccharide in nature as the capsule of *B. anthracis*, for example, consists of a polypeptide of the unnatural D-glutamic acid.

A second property which often distinguishes a virulent organism from an avirulent one is the power of the former to produce a "toxin." A toxin can be described as a substance which is secreted specifically by an organism, which produces general toxic reactions in the host and which gives rise to the production of specific antibodies in the host's blood-stream. When virulent organisms are injected, after an initial phase of establishment, they proceed to multiply and produce toxin. At the same time, the phagocytes mobilise around the site of entry, and one of the actions of the toxin is often to antagonise the phagocytic action and so impair the defence mechanism. The circulation of toxin in the host's blood-stream exerts the toxic action with the production of clinical disease. A further effect of the toxin circulating in the blood is to stimulate the cells of the bone-marrow to produce specific antitoxin, the chief function of which is to render the toxin ineffective. If the bone-marrow response is sufficiently rapid, then antitoxin is poured into the blood, the toxin neutralised, and the reticulo-endothelial cells once more enabled to attack

and phagocytose the invading organisms. If the toxin production is sufficiently powerful or the bone-marrow response too slow, then the toxic action may cause the death of the host as in Case 3. If the toxin production is moderate and the bone-marrow response adequate, then the initial advantage given to the organism by its toxin production is overcome and the invading cells eventually removed (Case 2). If the invading cells are avirulent or saprophytic and produce no toxin, then the reticulo-endothelial system is able to sterilise the host's tissues without difficulty (Case 1).

A pathogenic organism differs, then, from a non-pathogen in that it possesses the power to produce a toxin, enabling it in some way or other to gain an advantage over the defence mechanisms of the host. This is not the sole property necessary to produce a successful pathogen, as the property of forming a toxin cannot be effective until the organism has actually invaded the host's tissues. Consequently a second factor of pathogenic importance is the degree of " invasiveness," or capacity to penetrate the host's tissues.

" Toxin " and " invasiveness " are names of two properties of a pathogenic organism that can be more correctly described in chemical terms. This branch of bacterial chemistry is one which is only now being developed, and has not as yet reached a stage where generalisations can safely be made. Consequently we shall take a specific case of disease causation, namely, the production of " gas gangrene " by the infection of a wound with *Cl. welchii*, and endeavour to explain the pathogenicity on a chemical basis. This disease is chosen as example as it has been very intensively studied in recent years, so that we now understand more of the chemical nature of the pathogenic action of *Cl. welchii* than of any other organism.

BIOCHEMISTRY OF GAS GANGRENE

Gas gangrene is the name given to the clinical condition following the infection of tissue, usually through a wound, with *Cl. welchii* and certain related strict anaerobes. It is

characterised by liquefaction of the tissues around the wound and the appearance of bubbles of gas within the muscular tissue around the wound—hence the name "gas gangrene." The gas may accumulate to such an extent that the infected flesh crackles when handled. The local condition in the wound is accompanied by pronounced shock, fever, and collapse which, in the absence of therapeutic measures, is usually fatal.

Gas gangrene became serious during the fighting in Flanders in 1914-18, when it was, for a time, one of the major causes of death following wounding. Bacteriological examination of the wound and the wound exudate showed heavy infection with certain species of strictly anaerobic bacteria. The main organisms concerned are three Clostridia: *Cl. welchii*, *Cl. septicum*, and *Cl. oedematiens*, placed in order of importance. Other Clostridia, particularly *Cl. sporogenes* and *Cl. histolyticum*, were often found in association with these three; these are not themselves pathogenic, but it has been observed that gas gangrene infections where the pathogenic organism is accompanied by one of these non-pathogenic species, are considerably more dangerous and progress with greater rapidity than those where a simple infection of *Cl. welchii*, etc., exists.

The Clostridia live a saprophytic existence in the intestinal contents of many animals. Several distinct toxigenic types of *Cl. welchii* have been identified, of which some, types B, C, and D, are associated with diseases of young farm animals. *Cl. welchii*, Type A, the causal organism in gas gangrene, appears to lead a normal and harmless existence in the intestinal contents of man and animals. The organisms are voided with the faeces, and a certain proportion of them form spores in the unsuitable environment of field and soil. The spores can remain in the resting state on the soil for many years, if necessary, until they eventually fall into an environment suitable for vegetative existence when they germinate to form viable cells ready for multiplication. A suitable environment for germination is provided by the tissues of a

wound. Consequently, whenever a wound becomes contaminated with soil, dirt, or dust containing manure, there is a possibility of gas gangrene infection from Clostridium spores in the manure particles. When soldiers fighting on cultivated land become casualties, the probability of infection is high, and gas gangrene becomes a major hazard of war.

Cl. welchii is a moderately proteolytic, highly fermentative organism. When the viable organism begins to multiply in the tissues surrounding an infected wound, the following changes take place:—

1. Liquefaction of the tissues

This is due to the action of extracellular proteases attacking tissue proteins and breaking them down to their constituent amino-acids with the consequence that the tissue loses its structure and "dissolves." In particular, *Cl. welchii* excretes a collagenase, a proteolytic enzyme which hydrolyses the collagen of muscle-fibres, including the sarcolemma, with the result that the fibre-bundles disintegrate.

2. Production of gas

There are two main sources of the gas which appears in the infected tissue. First, the organism is able to ferment muscle glycogen with the production of hydrogen, carbon dioxide, acetic and butyric acids, and other products. However, if *Cl. welchii* is grown in the presence of meat protein freed from glycogen, gas is still produced in large quantities. This gas is produced by the deamination of certain amino-acids, especially serine, with the liberation of H_2, CO_2, NH_3, etc. (see p. 167). The action of the proteases on tissue protein assures a steady supply of free amino-acids to act as source of gas in this way.

3. Production of histamine

Cl. welchii, when growing in an acid medium, produces histidine decarboxylase. Histidine is liberated from tissue protein by the action of proteases, and the fermentation of

muscle glycogen leads to localised pockets of acid, consequently conditions are suitable for the production of histamine within the wound. The histamine content of the muscles of a cat may increase by 100-250 per cent. when *Cl. welchii* infection is established, but since it is not possible to demonstrate any increase in the blood histamine, it is doubtful whether this histamine production has any generalised effect.

The three occurrences so far outlined are the result of the simple metabolism of the organism and play their part in the superficial characteristics of the disease and its clinical picture, but are not seriously concerned in the lethal nature of the infection. When infection with *Cl. welchii* is accompanied by contamination with either *Cl. sporogenes* or *Cl. histolyticum* then these three factors so far discussed become exaggerated as both these *Clostridia* are highly proteolytic. Consequently their presence leads to a more rapid liquefaction of the tissues with increased supply of amino-acids to the pathogenic organism.

4. Invasion of the tissues

If a number of experimental infections of animals are made with various strains of *Cl. welchii*, it is found that whereas some organisms establish themselves in the wound quickly, penetrate the tissues, and set up a fulminating gas gangrene, others, though of equal toxicity, are unable to establish themselves or to penetrate the tissue. The strains are said to vary in their invasiveness. Penetration of tissue by bacteria is opposed by a barrier of highly viscous mucoprotein between the tissue cells, and many organisms are unable to penetrate such a barrier. Highly invasive organisms have the power to decompose the polysaccharide portion of the complex by the formation of an extracellular enzyme. The polysaccharide concerned in muscle tissue is called hyaluronic acid and consists of equal parts of glucuronic acid and N-acetylglucosamine. Some organisms excrete an enzyme called "hyaluronidase," which is able to attack and decompose hyaluronic acid. The chemistry of the breakdown is not yet

known in detail, and it is probable that hyaluronidase is actually a mixture of enzymes. The result of hyaluronidase action on hyaluronic acid is a great reduction in viscosity accompanied by hydrolysis and liberation of N-acetyl-glucosamine. The barrier to penetration is thus overcome by those organisms, including *Cl. welchii*, which can excrete hyaluronidase.

5. The production of toxins

It is not the proteolysis, gas or histamine production, or hyaluronidase action of *Cl. welchii* that eventually kills the host, but the production of toxins by the organism. It is possible to grow the organism in a suitable medium, filter off the organism, and kill a host animal by injecting the cell-free medium containing toxins elaborated by the organisms during growth. The medium can be concentrated and fractionated, and the toxin extracted in a highly purified state. It has all the properties of a protein and its toxic action is destroyed by heat.

By fractional precipitation the "toxin" of *Cl. welchii*, Type A, can be divided into two:

(*a*) α-TOXIN: this substance has three biological actions:

1. If it is mixed with a suspension of red blood corpuscles, it brings about haemolysis or disintegration of the cells and is consequently said to be "haemolytic."

2. In its presence, tissue cells disrupt and the toxin is said to be "necrotic."

3. If minute amounts are injected into an experimental animal, the animal dies within a few minutes, so that the toxin is "lethal."

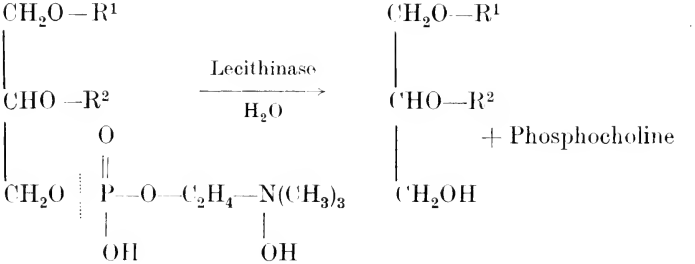
(*b*) θ-TOXIN: this is also haemolytic, but is neither necrotic nor lethal, except in comparatively large doses. The lethal properties of *Cl. welchii* reside mainly in the α-toxin.

In the past there have been two main theories concerning the possible nature of toxin action: (*a*) that toxins are enzymes

which interfere in some way with the essential metabolism of the host; (*b*) that they act in some other way by blocking an essential activity in the host.

MacFarlane and Knight have shown that preparations of the α-toxin possess the activity of a lecithinase, and that during purification, increase of toxicity is paralleled by increase of lecithinase activity so that the purest preparations of toxin are also the most active lecithinase preparations.

Lecithinase hydrolyses lecithin with the liberation of phosphocholine:



R^1, R^2 = fatty acid residues.

Lecithin is an essential component of the membrane of cell-walls, so that if the lecithin is decomposed, then the cell-wall disintegrates. If the lecithin of the cell-wall of a red blood corpuscle is hydrolysed, then the cell-wall disrupts and the red cell haemolyses. In the same way tissue cells disintegrate, and it is only reasonable to suppose that such a reaction, taking place generally throughout the body, would be lethal in its final effect. It is highly probable that the α-toxin of *Cl. welchii* is actually a very active lecithinase. Both α-toxin and lecithinase are inactive in the absence of calcium ions, and the lecithinase activity of the preparations is inhibited by the specific antitoxin.

The chemical action of the θ-toxin has not yet been discovered. It also acts on some substance in the wall of the red blood cell in a way that leads to disintegration but, assuming that it is likewise an enzyme, its substrate has not

yet been identified. The θ-toxin is active only in a reduced state and can be completely inactivated by oxidation; the nature of effective oxidising and reducing agents suggests that the group which is oxidised in the toxin molecule is —SH, oxidation to —SS— leading to inactivation.

We have attempted in this way to explain the toxicity of *Cl. welchii* by analysing the various factors which act biochemically in a way that we might expect would explain the toxic action. In the same way we endeavour to explain the oxidations carried out by bacteria in terms of the activity of isolated enzyme systems. When we come to integrate our findings with isolated enzyme systems in terms of the activities of the intact cells, we find that the interplay of various environmental factors, etc., complicate the reactions established *in vitro*. In the same way we find that we cannot explain the *complete* pathological picture found in gas gangrene in terms of the activities of the separate factors we have discussed. The exact importance of the role played by toxins in the clinical picture is not yet clear, and it has been suggested that some part may be played by substances released from muscle cells and necrotic tissues on disruption. There are almost certainly some factors brought into play by the interaction of the infecting organism and the infected host which have not yet been revealed by studies *in vitro*.

THE NATURE OF BACTERIAL TOXINS

The α-toxin of *Cl. welchii* is probably an enzyme whose substrate is an essential structural unit in the cells of the host. The pathogenicity of this organism depends largely upon its power to excrete certain enzymes which attack the tissues of the host as substrate. It does not, of course, follow that all toxins are necessarily enzymes, but such a hypothesis fits in with what is known of the nature of many of them We must now expect that work will be intensified with a view to establishing the enzymatic nature of other toxins and the identity of their substrates. The work is difficult as there is often no clue as to the possible nature of the substrate; with

Cl. welchii a clue to the nature of the substrate existed in the fact that when the organism is grown on serum-plates or in egg-media, the medium becomes turbid and this turbidity is due to the formation of minute fat droplets. We know now that these arise from the hydrolysis of lecithin in such media. Haemolysins, such as the θ-toxin of *Cl. welchii* and the toxins of *S. haemolyticus*, presumably act by degradation of a vital constituent of the wall of the red blood cell, but there seem to be no obvious clues to the chemical action of other toxins such as that formed by *Cl. botulinum* which is probably the most active of all exo-toxins.

The toxins of certain pathogens, such as *Cl. tetanum* (tetanus) or *Cl. botulinum* (botulism), seem to be very much more active than those of *Cl. welchii*, and it may be that they act as enzyme inhibitors rather than as enzymes themselves. It has been suggested that the toxin of *Cl. tetanum* is an inhibitor of choline esterase, but proof is not yet available. These two toxins of *Cl. tetanum* and *Cl. botulinum* have been obtained in a crystalline state very recently, so we may expect further developments in the near future.

THERAPY: THE COMBATING OF PATHOGENIC BACTERIA

At the beginning of this chapter it was shown that whether or not disease follows the contamination of a host's tissues with a bacterium depends upon the relative activities of the bacterium and of the reticulo-endothelial system. The aim of medical science is to prevent the organism gaining the final advantage in any infection or, referring back to Fig. 13, to reduce Case 3 to Case 2 and, if possible, to Case 1. The most successful ways of accomplishing this are based on two fundamental principles:

1. Immunological defence

A property of a toxin or any foreign protein in the blood-stream is to stimulate the formation of an antitoxin or anti-body by the bone-marrow. The chemistry of antigens and

antibodies is a specialised branch of the subject outside the scope of this book, but one obvious method of combating infection is to assist the production of the antibody against the infecting agent. This can be done in various ways, which have varying effectiveness against different organisms. In cases where the infection is already established and toxaemia present, it is sometimes possible to inject the specific antitoxin itself. Antitoxin is made by injecting sub-lethal doses of toxin into a large animal such as a horse, removing the plasma after antitoxin formation has occurred, and using some preparation of this plasma as a source of antitoxin. It is more satisfactory to produce antitoxin in the blood of the host itself and, if possible, to produce this prior to infection so that accidental contamination with the pathogen will not advance into virulent infection. Such " active immunisation " can often be accomplished by the injection of some harmless preparation of the toxin or organism and so stimulating the antibody response that the antibody concentration in the blood-stream will remain effective for some considerable time after immunisation. To stimulate such a response three main types of preparation are used: (1) a " vaccine " consisting of a suspension of organisms which have been killed either by heat or by chemical treatment and are consequently non-viable; (2) preparations of the toxin itself, injected in minute doses at first and then in increasing doses at intervals until a sufficient antitoxin response has been built up; (3) toxoid preparations—if the toxins of some organisms such as *Cl. welchii*, *Cl. tetanum*, *Corynebacterium diphtheriae*, etc., are treated with a weak solution of formaldehyde, their chemical structure is altered in some way which results in the destruction of their toxic nature but not of their ability to stimulate antitoxin formation; consequently, in these cases, it is possible to inject a relatively large amount of " toxoid " to stimulate a correspondingly large antibody response without any toxic effect on the host. All of these methods are used to combat specific infections and each has its advantages in certain conditions, but all are dependent

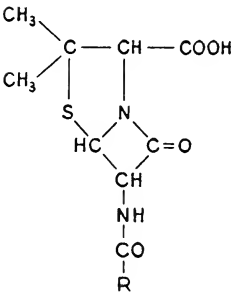
upon the antibody response being effective over a reasonably long period, as it is undesirable to repeat the treatment frequently.

2. Chemotherapeutic intervention

If the immunological method is ineffective or difficult, an alternative method of therapy is to prevent the growth of the organisms by chemical means. We must distinguish between bactericidal agents, which actually kill the organisms, and bacteriostatic agents which do not kill but prevent multiplication of the bacterial cells and so allow the reticulo-endothelial system to attack and remove the invaders. The main chemotherapeutic agents in use to-day are:

Natural antibiotics: substances produced by micro-organisms and which are naturally bactericidal or bacteriostatic.

1. PENICILLIN: a substance produced by various moulds, particularly *Penicillium notatum*. It is bactericidal in very high dilution against Gram-positive bacteria—*Staph. aureus* being inhibited *in vitro* by 1 part penicillin in 3×10^7 parts water or medium. Penicillin is exceptional in that it is non-toxic to man so that large amounts can be injected to deal with established or stubborn infections. One of its actions is to prevent the assimilation of glutamic acid, and possibly other amino-acids, needed by Gram-positive species for the synthesis of bacterial protein (see p. 99). Sensitive organisms continue to grow for a time after the addition of penicillin to the medium, but the growth produces abnormally large and distorted cells. After this short period of growth the cells become non-viable and eventually undergo lysis. Penicillin has no effect on the respiration of washed suspensions of *Staph. aureus*. The chemistry of penicillin has received intensive attention during recent years but there is still some doubt about its structure. There are several substances produced by moulds which have the properties of penicillin and differ in the chemical structure of the group R in the following formulae.

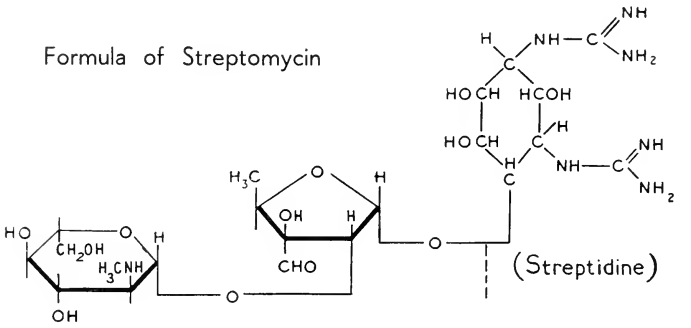


R =  -CH2— (Penicillin I)

= $CH_3 \cdot CH_2 \cdot CH = CH \cdot CH_2$— (Penicillin II)

Formula of Penicillin.

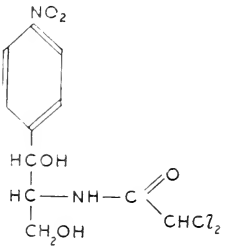
2. STREPTOMYCIN: a substance excreted by the mould *Streptomyces griseus* and active against both Gram-positive and Gram-negative bacteria. Its use in medicine is restricted since many pathogenic organisms rapidly acquire resistance against it. Streptomycin is the first antibiotic to be effective against the tubercle organism *in vivo* and it has been of great use in combating tuberculosis. Attempts are being made to extend its clinical usefulness by giving it in conjunction with some other drug such as a sulphonamide or sulphone which, by preventing multiplication of the organisms, will also prevent acquirement of resistance to streptomycin. The formula is given below. Streptomycin may act as a nutritional antagonist as its structure contains the unnatural analogue of glucosamine and a possible analogue of inositol. Its mode of action is not yet clear: experiments with intact sensitive cells suggest that it interferes with some stage in the oxidation of pyruvate. The stage affected is concerned with a reaction involving pyruvate and oxalacetate, possibly a condensation similar to that occurring in the citric acid cycle (see p. 153). However, it is not known whether this cycle functions in bacteria and no demonstration of an action of streptomycin on a cell-free bacterial system has yet been published.



3. Toxic peptides secreted by bacteria: *B. brevis* is a spore-bearing soil organism which secretes an antibacterial substance called tyrothricin. This is a mixture of peptides, the most important being Tyrocidin and Gramicidin. Tyrocidin is a surface-active substance which kills both Gram-negative and Gram-positive bacteria by dissolving lipoid materials in their cell-walls. Gramicidin is much less toxic and is bacteriostatic towards Gram-positive bacteria; it is thought to act by interfering with the assimilation and metabolism of phosphate. Both tyrocidin and gramicidin are too toxic for general clinical application, although purified gramicidin can be used locally in wounds. These two peptides proved to be the forerunners of a series of similar substances produced by bacteria, especially those of the genus *Bacillus*. Of recent years, antibiotics called Polymyxin, Aerosporin, Bacitracin, Subtilin, Bacillin, etc. have been described. They all seem to be polypeptide in nature and contain some unnatural D-amino-acid residues. Some are known to be cyclic polypeptides. They are stable substances and vary considerably in their antibacterial properties: bacitracin has a range of activities very similar to that of penicillin, while polymyxin is effective against organisms of the Gram-negative group which are comparatively resistant to penicillin. The clinical

application of many of these substances is still in doubt as their use is often accompanied by damage to the kidney tubules; whether this is caused by toxic impurities or whether it is a corollary of the excretion of peptide substances is not yet known.

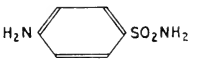
4. AUREOMYCIN, CHLOROMYCETIN: These substances are organic bases formed by species of *Streptomyces* and have a wide range of antibacterial activities. Their discovery is of great importance for several reasons: first, they are more stable than other antibiotics that can be used chemotherapeutically; second, they are the first substances to be effective against rickettsial and virus diseases; and third, the structure of chloromycetin is relatively simple and the active substance has been synthesised. The synthesis is not difficult and chloromycetin will probably be the first antibiotic which can be produced more cheaply by chemical synthesis than by biological production.



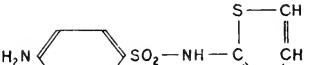
Chloromycetin

SULPHONAMIDES: various derivatives of *p*-amino-benzene-sulphonamide are used with considerable success against Gram-positive organisms, while some Gram-negative organisms are susceptible to the action of the more active derivatives such as sulphadiazine or sulphathiazole. Successful derivatives are those in which the sulphonamido-N group contains a substituent such as pyridine, pyrimidine, thiazole, etc.; the pyrimidine and thiazole derivatives have a relatively high solubility in blood-plasma and can be used for disinfection

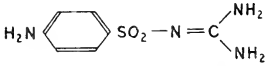
of the blood-stream. Sulphaguanidine is comparatively insoluble, is scarcely absorbed from the gut and consequently finds use as an intestinal disinfectant. The sulphonamides act by competing with *p*-amino-benzoic acid in some essential metabolic path in the organism (see Chap. V).



SULPHANILAMIDE



SULPHATHIAZOLE



SULPHAGUANIDINE

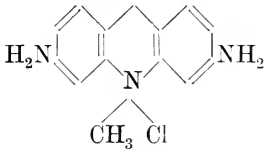
MARFANIL, ETC.: Marfanil is *p*-sulphonamido-benzylamine, but its action appears to bear no relation to *p*-amino-benzoic acid metabolism, as it is not antagonised by this substance and sulphonamide resistant organisms are sensitive to marfanil. It is more effective than the sulphonamides in the presence of pus, but is non-effective on injection, probably since it is decomposed by the amine oxidase of body tissues. Derivatives

Marfanil

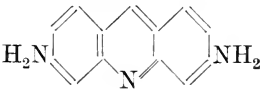
in which the amino- and amido-groups are substituted prove effective against the Clostridia.

ACRIDINE DERIVATIVES: certain mono- and di-amino-acridines, such as proflavin, acriflavin, etc., are active in high dilution against Gram-positive bacteria and, to a less extent, against Gram-negative organisms. They are relatively non-toxic to man. Since acridines combine with nucleotides, it is thought that these substances interfere with coenzyme systems of bacteria and so block certain metabolic paths. Their use, other than for superficial application, has been

largely discarded in favour of sulphonamides and penicillin.

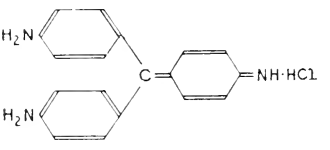


Acriflavin

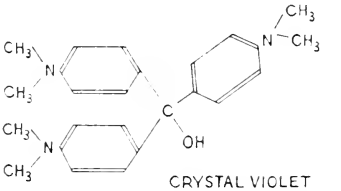


Proflavin

TRIPHENYLMETHANE DYES: mixtures of tetra-, penta-, and hexa-methyl-tri-amino-tri-phenyl-methane dyes are effective against some Gram-positive bacteria, but are also somewhat toxic to the host. They are commonly used in burn dressings, where their coagulant properties are of use.



p-ROSANILINE



CRYSTAL VIOLET (CARBINOL BASE)

The relative effects of some of these antibacterial substances when tested *in vitro* against a typically Gram-positive *Staph. aureus* and Gram-negative *Esch. coli* are given in Table XVI.

TABLE XVI

LIMITING EFFECTIVE MOLAR CONCENTRATION OF SOME ANTIBACTERIAL AGENTS

Concentrations expressed in $\mu M = M \times 10^{-6}$

	Staph. aureus μM	*Esch. coli* μM
Penicillin	0·03	300
Sulphathiazole	0·5	1
Crystal violet	0·3	30
Streptomycin	5	25
Aureomycin	10	100
Acriflavin	15	30
Sulphanilamide	60	20

All these values are subject to wide variations with the strain of the test organism and with the nature of the growth medium.

The effective dilution of any particular substance may vary considerably with the nature of the medium and conditions of test.

FOR FURTHER READING

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INDEX

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