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PHYSIOLOGICAL BACKGROUND TO MICROBIAL INHIBITION

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Ehrlich put forward the idea that the objective of chemical attack upon noxious micro-organisms should be to interfere with the metabolic processes of the parasite in a specific manner, so that the host itself be uninjured. At the time Ehrlich proposed this, however, relatively little was known about those metabolic processes which could be injured or about how they could be injured. The result was that, even with this guiding principle, there was little opportunity to apply it in practice, except indirectly and empirically. For instance, Ehrlich chose the objective of designing organometallic compounds whereby a substance such as arsenious oxide, already known to possess toxic properties for some biological forms, should be incorporated as part of more or less complex carbon compounds, so that the general toxic activity might be modified and made more specific. The various chemical structures were designed in a more or less empirical manner, based on the possibilities of organic chemistry rather than on an assumed or known mode of action defined in terms of cell-physiology. Modifications of structure were guided by the results of biological tests of inhibitory effectiveness and eventually by clinical trial. Thus the main practical result of Ehrlich's ideas in the guidance of research was related chiefly to the enhancement of specificity, guided by biological tests, when a lead as to toxicity had been obtained. Of course this line of approach did yield compound 606 (salvarsan) and certain antitrypanosome substances. But these studies were not paralleled by complementary studies of the physiology of the organisms concerned, that is to say, of the metabolic processes which were being affected. Biological testing and clinical trial do admittedly leap over certain intermediate stages in the development of a therapeutically-useful antimicrobial substance. A given compound may well be toxic at high dilution when tested in vitro against a given micro-organism in isolated culture, and yet may prove therapeutically valueless when used to attack that micro-organism in a biological environment such as a host animal.

Nevertheless, if there is to be a rational search for antimicrobial substances, it would seem logical to develop an understanding of the targets against which these substances are to be aimed, namely, the vital processes

of the parasite. In that way it is possible to define specific targets in terms of cell-physiology, to understand the mode of action of successful antimicrobial agents, and thus to be in a better position deliberately to design other possibly simpler or more effective inhibitors. It is also necessary to study the vital processes of the host. The host provides the battle-ground for the fight between microbe and microbial inhibitor and also takes part in the conflict.

Since Ehrlich's time great advances have been made in the knowledge of the vital processes of micro- and macro-organisms, but the development has been uneven; much greater progress has been made in some directions than in others. Also, it must be admitted that the discovery of the two new groups of chemotherapeutic antimicrobial substances—namely, the artificial sulfonamides and the naturally-occurring antibiotics, typified by penicillin—did not follow from a rational application of Ehrlich's conception in the light of modern knowledge of microbial physiology. Both these groups of chemotherapeutic substances were discovered empirically; in neither case was a specific physiological target aimed at.

It seems worth while to consider some aspects of the present position in order to be reminded of the many gaps in knowledge which still exist, and to see how the discovery of the sulfonamides and natural antibiotics bears on the problem of the rational design of antimicrobial substances. Short of understanding the detailed physiology of both parasite and host there remains, in effect, only the empirical attack : the discovery of effective antimicrobial agents by large-scale screening operations. Once some such agents are discovered it becomes possible to work back to discover their mode of action and thus to secure an insight into perhaps otherwise unrecognized but vital aspects of microbial physiology. Thus, in any case, for a rational approach to the design and improvement of antimicrobial agents, a fundamental requirement is detailed knowledge of the physiology of the organisms concerned. Some aspects of microbial physiology which bear on the problem of microbial inhibition will now be sketched.

Chemical Anatomy of Micro-organisms

The phrase " chemical anatomy " is intended to denote the materials of which the living cell is constructed, considered statically. This material is the end-product of the vital processes which lead to the production of new quantities of living organisms. The viruses, and the peculiar problems of biosynthesis which their mode of commensalism present, are omitted from the following generalizations.

Micro-organisms have as important components : proteins, constructed from some 20 amino-acids; nucleic acids, with their purine and pyrimidine bases; lipids; polysaccharides; and a number of carbon compounds of

peculiar and specific structure which appear to be universally distributed in the cells of all organisms, with the exception, mentioned above, of the viruses. This latter group of compounds includes those known as the water-soluble vitamins of the B group, when considered from the nutritional aspect, and as components of various enzyme systems, when considered from the functional aspect.

Analysis of the chemical components from the point of view of function yields other groupings :

(1) Structural materials which constitute the material form of the cell. There are, for example, cell-walls, cell-membranes, capsules, flagella, cytoplasm, nuclear material, indeed all the morphological elements revealed by cytology. Their chemical components include proteins, polysaccharides, nucleic acids, and lipids. Structural differences between the cells of different organisms may play very important parts in differential growth-inhibition, since they may affect accessibility of growth inhibitors to vital sites in host and parasite differently, and thus form a basis for selective toxicities.

(2) Materials which form the metabolic mechanisms. In this category, the characteristic biological materials known as enzymes are, of course, outstanding and essential. The cell enzyme-systems are the means whereby the essential chemical reactions which constitute the life of the cell (maintenance, growth and multiplication) are catalysed under physiological conditions.

These two categories (1) and (2) are, of course, closely interrelated and it would be difficult to say that any structural material was devoid of enzyme properties. Certainly for some cells it has been calculated that a very high percentage of the total protein can be accounted for as enzyme protein.

The enzyme systems can be grouped formally into two classes :

(*a*) those concerned in conducting the steps in the chemical syntheses of new cell-substance and the steps in the concomitant energy-yielding (exergonic) reactions;

(*b*) enzymes concerned with the syntheses of these enzyme systems themselves and any other enzymes needed in the economy of the cell.

Little is known about protein synthesis and practically nothing about the conferment of specific protein characters. More is known about the biosynthesis and metabolic interrelations of the constituent amino-acids of protein, in terms of the chemical steps involved, but practically nothing about the enzyme control of these chemical reactions. Thus the component building-blocks of protein are known, i.e., the 20 or so L-α-amino-acids, and some chemical steps in their biosynthesis, but little else about the biosynthesis of this material of cardinal biological importance.

A little more is known about the synthesis of the nucleic-acid portion of nucleoprotein which, as an essential component of nuclear material, plays

an important role in growth and in the determination and specificity of daughter cells.

The most detailed present knowledge of the chemical composition and biosynthesis of cell enzymes concerns the prosthetic groups of a number of enzyme systems concerned with the energy-yielding (exergonic) reactions of carbohydrate metabolism and some others whose physiological function is less clear though apparently essential under natural conditions.

Many of these enzyme components were discovered because an inability of certain organisms to synthesize them results in the possibility of revealing their existence in terms of a nutritional deficiency. This is the group of water-soluble B vitamins and microbial growth-factors. A good deal is known about the biological routes of synthesis of a number of these compounds, something is known about the metabolic functions of the enzyme systems of which they form parts,[a] but, again, almost nothing is known of the enzymes which take part in the biosynthesis of these enzymes.

The foregoing formal analysis gives some of the main categories of material which have to be reproduced in cell growth and multiplication and which form the essential end-product of the organism's metabolic processes.

Physiology of Micro-organisms

When the microbial cell is considered from the point of view of the metabolic reactions it carries out, it is evident that these constitute to a very large extent its whole life-process. With bacteria it is convenient to distinguish between " growth " in the sense of increase of bacterial protoplasms, and " multiplication " as increase in the number of viable units since, at least under some conditions, these can be separated. The metabolic processes of growth, i. e., the sheer increase in amount of cell-substance, constitute a very large proportion of the total metabolic effort, at least from the anthropocentric point of view. The metabolic processes carried out by a micro-organism can be subdivided into several formal categories :

1. The chemical synthesis of new cell-substance, i.e., the building up of the complex components of protoplasm from the less complex components of the nutrient environment.

2. The exergonic reactions needed to cause the endergonic syntheses to occur.

3. " Preparative " metabolic processes.

4. Syntheses of characteristic metabolic products.

These categories may be considered a little more fully since it is here that more specific definition of possible targets for antimicrobial agents is approached.

[a] See paper by Woods on page 35 in this number of the *Bulletin.*

1. *Synthesis of cell-substance*

The nutrient environment provides the chemical elements and compounds which have to be chemically synthesized and biologically integrated into new living cell-substance. The degree of chemical synthesis that may be necessary varies with the physiological character of different organisms. It may be as extensive as in those photosynthesizing and chemosynthesizing autotrophic organisms which can fix carbon dioxide as starting-points for the synthesis of the most complex components of their protoplasm, and which use light energy or energy from chemically simple specific oxidations of certain inorganic compounds. Or it may be much less extensive, as in those bacteria, such as *Clostridium* and *Lactobacillus* species, which require as nutrients an array of amino-acids, several growth-factors, and carbohydrate as energy source.

The new cell-substance made represents thermodynamically a local increase of free-energy, to compensate which a corresponding decrease of free-energy must occur in the cell's environment. The energy for the endergonic syntheses is that provided by the metabolic reactions of the next category.

2. *Exergonic reactions*

Much is known about the chemical steps in the exergonic reactions of carbohydrate breakdown which are exploited by many heterotrophic micro-organisms, but much less is known about the exergonic reactions used by the photosynthesizing and chemosynthesizing autotrophs.

The metabolic processes of categories 1 and 2 are closely interlinked. For example, the transfer of energy from the exergonic carbohydrate degradations to the endergonic biosyntheses in heterotrophs takes place through the material transfer of some energy-rich compound or radical. These two categories may be called essential metabolic processes in that, when they cannot operate, growth does not occur. The interlinked reaction-chains of chemical synthesis and energy-production are cardinal, and other chemical reactions which a cell may carry out are, in this sense, secondary. The essential metabolic reactions leading to the production of new cell-substance provide the necessary material basis for the performance of biological functions such as nuclear division, cell-division, and formation of capsules, flagella, antigens, and other special metabolic products. The next two categories of metabolic processes (3 and 4) are not essential in all circumstances, as are categories 1 and 2, although they may be in some circumstances.

3. " *Preparative* " *reactions*

These reactions may be so named in the sense that they prepare substances for use in reactions of categories 1 and 2, as, for example, in breaking

down a protein to amino-acid fragments or a polysaccharide to component hexoses. A preparative metabolic process may thus be less or more important, depending on the composition of the nutrient environment. These reactions are often responsible for characteristic physiological properties by which given organisms are commonly recognized. They are not necessarily essential for growth under all conditions, though they may be under some, particularly under natural, conditions.

4. *Production of specific non-essential metabolic products*

This category is not simple to define except by examples of the kind of product, e.g., specific antigens (exotoxins, exocellular enzymes, flagellar antigens) and natural antibiotics. These characteristic and important products are formed under less or more restricted sets of environmental conditions, represent a considerable degree of chemical synthetic effort, and are not in general the result of degradative transformations of material of the environment. In many cases it would appear that the formation of this class of metabolic product is not a necessary consequence of growth and multiplication, since organisms can often be cultivated under conditions where the given metabolic product is not formed. Nor is the production of such compounds always necessary for the life of the organisms which can make them. To what extent parts of the chains of synthetic reactions leading to these metabolic products may be necessary for the life of the cells which can produce them, is another question. The metabolic products themselves may be due to a diversion or distortion of parts of essential biosyntheses in some cases. But it would appear clear that at least the final steps leading to the characteristic product, e.g., a specific antigen, are not essential to the life of the given organism, at least when growing isolated in pure culture. To what extent this may be true, however, when looked at from a wider biological angle, is a more open question. It is clear that under natural conditions metabolic products of category 4 may have important survival value for the organisms which can produce them. Some knowledge of the effects of such metabolic products in competition between organisms under natural conditions, as among the soil microflora, is already available, and the possible general significance of such products can easily be imagined.

The foregoing formal categories of metabolic processes cover most of the physiological activities of micro-organisms and define, to some extent at least, a physiological target for growth inhibitors. This target needs further definition, both as to the specific metabolic reactions themselves, and as to ways in which they can be stopped.

Essential Biosyntheses

It is convenient next to consider those aspects of microbial physiology which are most clearly defined—namely, the essential biosyntheses. These have at present the most bearing on the topic of growth inhibition because it is here that the question of the mode of action of inhibitors has been most deeply investigated. This is the field where at present most is known both about potential metabolic targets and about the means whereby they can be deliberatively attacked. Some of the essential components of microbial growth-processes have been revealed through nutritional studies. There was a long period of study of the nutritional requirements of certain micro-organisms, extending back to the end of the 19th century and thus beginning before the deliberate study of animal vitamins; these studies of microbial nutrition continued parellel with animal studies for a number of years without known direct connexion. It was not until animal vitamin studies had led to the isolation of pure vitamin B_1 (aneurin) that wider aspect of nutritional studies could be recognized. Then it was found that vitamin B_1 was also a growth-factor required by various micro-organisms, and the reciprocal significance of microbial studies could be recognized.

The first indication of a mode of physiological function of growth-factors and vitamins came with the recognition that riboflavine was a component of Warburg's yellow enzyme, and later that nicotinic acid, already known as a component of co-enzymes I and II, was a nutritional essential for certain macro- and micro-organisms. There followed the identification of further specific microbial growth-factors, which were soon found to be implicated also in the nutrition of other organisms. Eventually there became known a number of " growth-factors ", one or more of which were required as nutritients by many different organisms of a wide range of physiological and biological types. These substances include : aneurin, riboflavine, pantothenic acid, pyridoxine, biotin, and nicotinic acid.

The next theoretical advance was the recognition of the general metabolic role of these compounds. This came through the recognition of the wide natural distribution of enzyme systems of which some of these compounds were essential components, and through the observation that where these compounds were not nutritional requirements for given organisms they were in fact synthesized by those organisms. Whether or not a component of one of these almost universally-used metabolic reactions appeared as a nutritional requirement, for a given organism, thus depended on the ability of that organism to synthesize the compound. Nutritional requirements therefore reflect biosynthetic deficiencies.[20] The term " essential metabolite " was introduced by Fildes[8] to emphasize the general metabolic role of many of those substances first discovered as essential nutrients

for particular organisms. These essential metabolites, then, are substances involved at each stage in any synthesis necessary for growth and without which, either synthesized by the cell or supplied from outside the cell (as a nutrient), growth cannot occur. Thus the discovery of essential nutrients is one way of discovering substances involved in essential metabolic reactions. The biological material with which such nutritional studies can be conducted has been much enriched by the development of the use of induced biochemical mutants of *Neurospora* and other micro-organisms, due to the work of Beadle, Tatum, Fries, and others. By these means the supply of organisms showing interruptions in the chains of biosynthetic reactions, as revealed by the evocation of new nutritional needs, is no longer dependent on the discovery of naturally-occurring strains with peculiar nutritional requirements.

These methods ultimately hinge on the possibility of revealing an interruption in a biosynthetic reaction-chain, induced by biological or chemical methods, by means of a growth response when a compound which is utilized in the biosynthetic chain, after the stage of interruption, is offered to the organism.

Two generalizations arise from the complementary studies of microbial nutrition and the biosynthesis of essential metabolites : (*a*) there appears to be a universal utilization of a number of metabolic processes, e.g., those that use the enzyme systems in which riboflavine, co-enzyme I or II, and aneurin function; (*b*) there appears to be a universal use of the same chemical steps in the biosynthesis of a given essential metabolite in such parts of that biosynthesis which different organisms are able to carry out.

It is as if certain routes of biosynthesis were first evolved by some primordial organism before a subsequent differentiation of organic forms took place, and that when these latter had evolved they then suffered varying degrees of loss of biosynthetic ability, leading to the evolution of the present nutritionally-exacting forms. The gaps in biosynthetic ability which different organisms, strains, or clones display are different, and hence their nutritional requirements are correspondingly different; but these are gaps in what appear to be commonly-used biosynthetic routes.[21] Thus nutritional studies in the broad sense give information about : (*a*) master compounds which function in essential metabolic processes, sometimes as components of essential enzyme systems; and (*b*) the chemical routes whereby these master compounds are biosynthesized. In this way possible targets for interference by antimicrobial agents are sharply defined, though only, it must be admitted, over a small part of the potentially attackable metabolic field. Nevertheless it is in this field that the sulfanilyl group of chemotherapeutic drugs has achieved its success.

Antimetabolite Growth-Inhibitors

The initial impulse which led to the conception of deliberately designing antimetabolite growth-inhibitors came through the impact of the therapeutic success of prontosil, and the demonstration that the potency of this complex molecule was due to the sulfanilamide part of it,[37] at a time when the general conception that nutritional requirements reflected deficiencies in biosynthesis had been mooted, and illustrated by the work of the Department of Bacterial Chemistry of the British Medical Research Council. Probably the first printed reference to the possibility that growth inhibition by sulfanilamide might be due to an interference with some growth-factor, essential for the inhibited organism, was made by McIntosh & Whitby.[25] Stamp,[34] working on a similar hypothesis, obtained extracts of streptococci which could overcome sulfanilamide inhibition. He pointed out that the active material appeared to be a normal cell constituent and might be the metabolite with which the action of sulfanilamide was concerned. As is well known, Woods[42] succeeded in identifying the metabolite in question which proved to be *p*-aminobenzoic acid (PAB). Until then, this simple actor in hundreds of lectures on elementary organic chemistry had not been suspected as capable of playing any important biological role. The same may also be said of sulfanilamide which was made by Gelmo[15] in 1908, 27 years before its chemotherapeutic action was observed.

The prediction by Woods[42] that PAB was an essential metabolite was verified when it was shown to be an essential nutrient for *Clostridium acetobutylicum*[32] and, subsequently, for certain other bacteria.

Much later, the determination of the structure of the independently discovered bacterial growth-factor, folic acid (pteroylglutamic acid), of which PAB forms part, substantiated the predicted essential metabolic role of PAB by revealing at least one reason for its importance in metabolism—namely, as a component in the biosynthesis of folic acid. In his paper, Woods[b] discusses the mode of action of sulfanilyl compounds in relation to interference with the biosynthetic utilization of PAB.

The discovery of the PAB/sulfanilamide relationship focused attention on the mode of action of the inhibitor because, as Woods showed, the inhibition of growth of streptococci by sulfanilamide and its annulment by PAB was of the same form as the competitive inhibition of an enzyme by a substance structurally related to its substrate. The generalized theory that other growth inhibitors might be analogously modelled by suitable modifications of other essential metabolites was put forward by Fildes.[8] This generalization, for a " rational approach to chemotherapy ", catalysed a large amount of investigation along those lines, and hundreds of structural analogues of known essential metabolites, including many of the natural

[b] See paper on page 35 in this number of the *Bulletin*.

amino-acids, have now been made. Many of the essential metabolite analogues are good and specific inhibitors of microbial growth when tested in vitro against pure cultures of micro-organisms and have proved valuable tools in the investigation of biosynthesis. However, from the point of view of chemotherapy, the harvest has so far been meagre, and apart from the various derivatives of sulfanilamide which show improvements on the parent compound, none of the other synthetic antimetabolites has proved of real therapeutic use in man.

Some reasons for this may be suggested. As already mentioned, the microbial essential metabolites are also essential metabolites for many other organisms, including man; they appear to be almost universally used in the construction of living matter. Thus interference with the use of an essential metabolite by an invading micro-organism may also interfere with its utilization by the host animal, or the amounts of metabolite present in the tissues of the host may overcome the effect of the antimetabolite on the invader. These are inherent difficulties in the design and use of antimetabolites as therapeutic agents. Clearly, though, this does not condemn all antimetabolites, as witness the usefulness of the sulfonamides, but it does impose a limitation which must be taken into account. It may well be that, in the long run, the most useful contribution of the antimetabolite theory will be the stimulus it is giving to lines of research which have yielded, and will continue to yield, information about the intimate physiology, in particular the basal biosyntheses, of many different kinds of organism. At least indirectly therefore, this should help in the discovery of new and better chemotherapeutic agents, since we shall be in a correspondingly better position to study modes of action and thus to design better inhibitors.

Although there is this basal common biochemistry of essential metabolites which function in both bacterial parasite and host animal, there may be other differences between the two biological forms which may assist in obtaining differential or selective effects even with antimetabolite types of inhibitors. Thus certain enzymes may be generally dispersed in the cytoplasm of a bacterium but located on some more specialized structures, such as mitochondria, in cells of the host. Enzymes thus located in different cytological structures may have different degrees of accessibility to inhibitors. It is quite possible that in this kind of cytological differentiation of sites of biosynthesis may lie one way of escaping apparent limitations imposed by a common biochemistry.

Biological Superstructure

It is on the basis of the fundamental biosyntheses which provide new quantities of protoplasm that the more biological aspects of cellular life, such as special cell structures, and mechanisms of differentiation and

multiplication, are grounded. The biochemistry of the processes involved at this biological level is much less well understood than the basal biosynthetic metabolism. It may well be that it is at this biological level that many of the natural antibiotics operate in causing growth inhibition; at least it seems clear that they do not act by direct interference with the utilization of known essential metabolites. The latter do not reverse the inhibitory effects of penicillin, aureomycin, or streptomycin, for example. Knowledge is meagre of the biochemistry of such biological processes as cell-division, division and aggregation of nuclear material, and the formation and germination of microcysts and spores. The situation is being remedied, partly by taking advantage of present possibilities of growing representative organisms in chemically-defined culture media. This makes it possible to study the biochemistry of the lag phase, of the initiation of growth and multiplication, and of biological processes such as those previously mentioned, under better-controlled conditions than when chemically-defined media cannot be used.

Morphological studies of the effects of penicillin and other substances on growing cells show that it is possible, at least to some extent, to separate the processes of sheer protoplasmic increase from those of cell-division, since the mass of protoplasm can increase without division occurring, resulting in the wellknown large forms. There are other ways of doing this, not involving the use of penicillin.

The inverse can also be done, i.e., the induction of cell-division at a rate which far outstrips protoplasmic growth. For example, the author has seen suitably oxidized spores of an obligate anaerobe (*Clostridium tetani*) maintained in the absence of free oxygen in a medium which was adequate except for the absence of free thiol (sulfydryl) groups (although disulfide groups must have been present). These spores were quite unable to germinate. But when some thiol compound was added, e.g., cysteine or reduced glutathione, these spores quickly germinated and subsequent cell-division was so rapid that it far outran protoplasmic increase. Instead of normal-sized rods the organisms looked like piles of coins, the cells in the chains being very much shorter than their width, which was practically normal; there was thus very little protoplasm in each unit.

The multiple physiological roles of biological thiol groups and thiol compounds have received considerable attention. It is interesting, historically, that the mode of action of a selectively toxic agent was first explained in chemical terms by Voegtlin[38] in relation to the mode of action of organic arsenicals,[1] appropriately enough, the type of chemotherapeutic compound of which Erhlich was the progenitor. It was shown by Voegtlin that the activity of arsenobenzenes was due to their oxidation to arsenoxides which then reacted with essential thiol groups in the parasitic trypanosomes. The arsenoxide level of oxidation is the only one at which the arsenicals are therapeutically active.[1] It is probable that various mercurials (e.g., mercuric

chloride, phenylmercuric nitrate) exert their inhibitory effect on the growth of bacteria, for example, also by combination with essential thiol groups of the inhibited organisms.[9] The development of the dithiol, 2,3-dimercaptopropanol (BAL), as an antidote for poisoning by arsenicals and various metallic poisons followed from studies of the mode of action of the poisons, namely, interference with the functioning of essential thiol groups. Other substances besides mercurials and arsenicals can inactivate thiol groups; for example, oxidizing agents (e.g., iodine); alkylating agents (e.g., iodoacetamide); arylating agents (e.g., quinones, quinonimines); aldehydes.

Cavallito et al.[3] suggested that the mode of action of a wide group of inhibitory agents, including penicillin, clavacin, and other natural antibiotics, was through interference or combination with essential thiol groups, because the inhibitory action could be abolished by cysteine, glutathione, and other thiols (as well as by ascorbic acid). Krampitz & Werkman[22] pointed out that inactivation of thiol groups would not appear to account for the specificities of action of various antibiotics which are in fact observed. Nevertheless, further analysis of the various specific physiological roles of thiol compounds in cell-division and in spore germination does appear worth while, since here at least is one more specific chemical grouping which is involved, though it is not known how directly.

Chemical requirements for the germination of spores of some species of *Bacillus* were studied by Hills[18] who found that L-alanine, L-tyrosine, and adenosine were important stimulators of germination. Only L-alanine was important for *B. subtilis* and its effect could be annulled by the D isomer in the surprisingly low molar ratio of 30D to 1L. Germination is a complex process, however, as indicated for example by the work of Powell[30] who showed retardation of germination of *B. subtilis* spores by 8-hydroxyquinoline and BAL which was partially reversed by ions of zinc, magnesium, iron, and copper, thus indicating that metals were involved somewhere in the process. The work of Albert and his co-workers,[31] on the effects of various metal-binding organic compounds and their studies of specificity, shows how subtle yet essential may be the biological functions of " trace " quantities of various metals in the economy of various organisms.

Webb's studies[39, 40, 41] of the effect of magnesium on cell-division in various bacteria, which showed that, at sufficiently low magnesium concentration, cell-division was impeded but not protoplasmic increase, so that long filamentous forms were obtained, reveal another aspect of the biochemistry of this biological process. These various effects of very small concentrations of metallic components of cell economies suggest again possible sensitive sites for selective toxic attack. These few examples are enough to indicate the present turning of attention to the chemistry of biological processes which are above the level of sheer protoplasmic syn-

thesis, and interference with which might be equally important in inhibiting the proliferation of a noxious organism as interference with its essential biosyntheses.

The mode of action of penicillin has been studied by Gale and co-workers.[12, 13, 14] It was shown that one of the results of the action of penicillin on growing cells of *Staphylococcus aureus* was to stop a metabolic mechanism by which glutamic acid was transferred from the environment through the cell-wall into the cell. This glutamic-acid import mechanism is endergonic and energy can be supplied by a concomitant glucose metabolism. Gale & Rodwell[14] showed that there was a correlation between penicillin-resistance and the ability of resistant strains to synthesize glutamic acid, an ability which thus rendered them independent of the glutamic-acid import mechanism. This proposed mode of action of penicillin is concerned with access of an essential metabolite, glutamic acid, to an internal site of protein synthesis. The role of the cell-wall and the endergonic mechanism for glutamic-acid transport to the interior of the cell are concerned with biological structure, and interference with their functions would be an example of interference with growth at a biological level.

Gale's hypothesis was criticized by Mitchell & Moyle[27] who pointed out that the component of the sensitive cell with which penicillin initially combines is unlikely to play a direct part in the glutamic-acid import mechanism since, as Gale showed, penicillin does not affect this mechanism in resting cells, and that there must be one or more intermediate steps in penicillin action before this mechanism is damaged. Mitchell & Moyle[27] studied relationships between cell-growth, surface properties of the cells, and nucleic-acid production in normal and penicillin-treated *Staphylococcus aureus*, and from their results criticized Gale's hypothesis. One of the most interesting of their findings was the discovery of a new form in which phosphorus was bound and which had, until then, been included in estimations of ribonucleic acid (RNA) by the method of Schmidt & Thannhauser[33] as modified by Stephenson & Moyle.[35] By this method a large proportion (approximately 30%) of phosphorus in excess of that belonging to nucleotide units of true RNA was estimated; this excess phosphorus really belongs to a polyolphosphate ester, XP, at present unidentified.[28, 29]

From Mitchell & Moyle's results it appeared that there was some interference by penicillin with the formation of the compound XP before the formation of RNA was affected.

"The inactivation of the glutamic acid accumulation mechanism in Gram-positive organisms may be associated with the changes in the cell surfaces, but the swelling effects which are known to occur in most organisms exposed to lethal concentrations of penicillin, whether or not they accumulate glutamic acid . . ., suggest a more generalized disturbance of the osmotic and mechanical functions of the cell envelopes than that which would only involve amino-acid accumulation."

Mitchell & Moyle conclude :

" . . . It is therefore impossible at present to decide which of the observed changes may be more closely connected with the initial disturbance. The evidence at present available suggests, however, that the disturbance of the ' free nucleotide '/nucleic acid balance and of XP production, and the changes in the cell surfaces, all precede the disturbance to the glutamic acid accumulation mechanism in *M. pyogenes*."[27]

In thus touching upon some investigation concerned with the mode of action of penicillin, all that it is wished to do here is to indicate that at present it seems that the site of action is not in the biosynthesis of the essential metabolic mechanisms in which the B-group vitamins participate. It may be concerned with the synthesis of nucleic acid or the intriguing compound XP; or it may be concerned with direct biochemical interference with some other biological function above the biosynthetic level.

Viruses

At the beginning of this paper, the viruses were specifically excluded from certain generalizations because of the peculiar problems presented by their commensal mode of existence. Not only is their chemical anatomy much less well known than that of other micro-organisms, but also very much less is known about their biosynthesis. The problem of bacterial growth is concerned with the synthesis and assembly of the cell's components by means of enzymes. But the growth of viruses has up to the present been shown to occur only in an already ordered enzymic environment, as in living cells. This is the peculiar commensal mode of existence and growth of viruses. The problem of their growth requirements therefore has to include consideration of the extent to which the enzymic functions of virus synthesis are carried out by the host cell or by the virus particle itself.[4]

Reference may be made to Laidlaw's conception[23] that the viruses are specialized parasites which have lost large degrees of synthetic ability and have become dependent on the synthetic ability of host cells to supply materials for the fabrication of new virus particles. The bulk of the synthesis is thus assumed to be done by the host cell, so that much of the metabolic mechanism of a free-living cell can be dispensed with, and the reproduced unit is stripped to its bare essentials—to the bare essentials of a particle which can reproduce and maintain its biological specificity. This theory of Laidlaw's thus posed the problem of virus growth in terms susceptible of biochemical attack.

Interesting work along this line is that of Cohen[4] who studied the nutritional requirements of host bacteria in relation to phage multiplication. He summarized evidence which led him to the conclusion that in some *coli* phages the greater part of the virus, namely its " polymeric structure of nucleic aid and protein " is synthesized by the enzymes of the host bacte-

rium " according to the bacterial metabolic relations of energy supply and assimilation of nitrogen, carbon and phosphorus . . . into new types of end-products whose specificity is determined by portions of the infecting virus particle ".[4]

Interesting results were obtained in the study of the effect of components in the nutrient medium of the host *Escherichia coli* on the multiplication of infecting *coli* phages. Strains of *Esch. coli* which can synthesize all the complex building blocks essential to bacterial and virus synthesis from simple nutrient components, e.g., ammonia and lactate, were used to study phage reproduction.[11] In such a simple chemically-defined medium, the rate and quantity of virus production was limited, there was a markedly longer latent period after infection, and the burst size was decreased, as compared with the behaviour in a nutritionally-rich medium, such as broth. The assumption was made that compounds present in broth and absent from the simple medium were responsible for the differences in virus growth. On the simple medium, stimulation of virus growth was obtained by addition of certain amino-acids (L-phenylalanine, L-aspartic acid, L-proline, L-lysine, L-valine, L-arginine, and L-glutamic acid). L-Glutamic acid produced the greatest effect; guanosine and deoxyguanosine also stimulated virus production. But no single compound, of some 60 common nutritional factors tested, reproduced the latent period and burst size observed in broth. Inhibitory effects were observed when L-leucine, L-serine, or L-cysteine were added. Inhibition by L-leucine could be overcome by the simultaneous addition of valine, isoleucine, or norleucine. These inhibitions by certain amino-acids and their annulment by other amino-acids are extremely interesting in view of similar amino-acid antagonisms in the growth of *Bacillus anthracis* observed by Gladstone,[16] where inhibition by leucine was annulled by valine, or vice versa, and inhibition by isoleucine or norleucine was annulled by valine plus leucine, or vice versa. That is to say, with *B. anthracis* the composition of an adequate nutrient medium with regard to its amino-acid content was a function not only of the presence of certain amino-acids which this organism could not synthesize, but also of the ratios in which certain amino-acids were present. An apparent nutrient " requirement " might be due not to an inability to synthesize that particular amino-acid, but to its presence being required to " neutralize " or balance the presence of another. The mechanism of this effect, which could be shown at very high dilutions of the reciprocal groups of amino-acids, remains unexplained, but may be envisaged in terms of competitions between amino-acids for specific enzyme sites concerned with the synthesis of specific proteins. The fact that similar antagonisms involving the same amino-acids have been shown in phage reproduction, as well as in bacterial growth, suggests that the phenomenon may be of general importance in connexion with protein synthesis.

Fowler & Cohen[11] found that a mixture of amino-acids, purines, and pyrimidines added to the basal ammonium lactate medium restored the latent period and burst size to that shown in broth, and used this to study virus synthesis, by omitting single components from the complex defined medium.[6] It was then found that omission of tryptophan or leucine markedly decreased the burst size without affecting the latent period.

" In parallel experiments with multiply-infective cells the time of onset of synthesis of DNA [desoxyribose nucleic acid] was delayed when these compounds were omitted but the rate of synthesis of DNA, once started, was that of the control. In these experiments it was found that L-glutamic acid, L-histidine, L-leucine, L-methionine, L-phenylalanine, L-tryptophan, L-valine and adenine are compounds which should be present in the medium for maximal production of virus."

Cohen & Fowler[5] studied the action on phage T2 synthesis in *Esch. coli* of the antimetabolite, 5-methyltryptophan, which is a specific competitor of tryptophan in protein synthesis.[10] 5-Methyltryptophan inhibits bacterial multiplication without inhibition of oxygen consumption or change of respiratory quotient, and does not inactivate $T2r^+$ phage or interfere with its adsorption to *Esch. coli*. At low bacterial concentrations, 5-methyltryptophan at low molar concentrations prevented phage growth when added to the medium before infection by phage, or at any time up to half the latent period, when tryptophan was absent from the ammonium-lactate medium. Tryptophan overcame this inhibition of phage growth by 5-methyltryptophan and permitted resumption of phage multiplication. Apparently 5-methyltryptophan stops a process which requires tryptophan within the first minute after infection of the host bacteria under these conditions. As Cohen[4] pointed out, this type of study could be extended with the use of sufficiently specific antimetabolites to other compounds thought to be important in phage multiplication.

The importance of nucleic acids in the composition of viruses has of course drawn attention to the use of inhibitors which might interfere with the synthesis of purines, pyrimidines, and their nucleotides. The work of Hitchings,[19] Thompson,[36] and co-workers is a good example of the attempt to obtain virus-inhibitory substances by the construction of analogues and derivatives of purines and pyrimidines which might be antimetabolites.

Pleuropneumonia-like Organisms

There is one more group of micro-organisms to which reference may be made, since some are pathogenic, and agents which inhibit them would be useful. Furthermore, their size and some of their properties put them in a class intermediate between the true bacteria and the viruses. This is the group of so-called pleuropneumonia-like organisms or *Borrelomy-*

cetaceae. Some organisms of this group of filterable organisms were found by Laidlaw & Elford[24] in sewage and were cultivated in cell-free media of the ordinary bacteriological type as long ago as 1936. Since then, numerous other organisms of this group have been cultivated in cell-free, but complex, undefined media. Their possible relationship to the L-forms of various bacteria also makes them of considerable biological interest. For a long time it was thought that these organisms required serum or ascitic fluid as a component of their nutrient media, and attempts were made to analyse the components of the complex media on which they can grow, but until recently little success was achieved. In 1951 a surprising advance was made by Edward & Fitzgerald[7] who found that the serum component of the usual media for cultivating a whole group of pleuropneumonia-like organisms could be replaced by an ethereal extract of egg-yolk. Fractionation of this extract suggested that cholesterol was an active component of it. Cholesterol alone did not replace serum but cholesterol plus the acetone-insoluble fraction (AIF) of the egg-yolk extract did. Purified preparations of kephalin and lecithin fractions from the AIF material were inactive. Cholesterol plus starch, or bovine serum albumin, or the unknown moiety (i.e., not lecithin or kephalin) of the acetone-insoluble egg-yolk material replaced serum in the media. Since starch, serum albumin, or AIF without cholesterol were ineffective, evidently cholesterol is needed for growth, even if the role of the other components is not yet clear.

This finding that cholesterol is required in the growth of a whole group of these organisms is very interesting because, up to the present, this substance has not been observed as a growth-factor for any bacteria, while it has been found necessary for a species of flagellate protozoon, *Trichomonas columbae*,[2] and for certain entozoic amoebae (*Entamoeba histolytica*, *Entamoeba terrapinae*)[17] and is important in the nutrition of various insects.[26] That cholesterol should be a growth requirement of organisms of the pleuropneumonia group, while no such observation has been recorded for any bacteria, is particularly interesting in view of the possible relationship of the former to the L-forms of bacteria. These recent findings of Edward & Fitzgerald appear to mark a real advance in the knowledge of the biochemistry of the pleuropneumonia group and are a considerable step towards growing these organisms in chemically-defined media. The possibilities of studying their biosyntheses, of interfering with these processes, and of studying the mode of action of naturally-occurring or synthetic inhibitors which may be found are therefore now much increased.

* * *

An attempt has been made to indicate some of the physiological background which is relevant to the design of microbial inhibitors and

to the study of their modes of action. Particularly it is wished to stress the value of studies of comparative biochemistry in this respect.

SUMMARY

Since Ehrlich first suggested that the objective of chemical attack on noxious micro-organisms should be to interfere with the metabolic processes of the parasite in a specific manner, leaving the host itself uninjured, great advances in the understanding of this subject have been made. It must be admitted, however, that detailed knowledge of these metabolic processes has developed further in some directions than in others, and that the discovery of the two important groups of therapeutic antimicrobial substances—namely the sulfonamides and the naturally-occurring antibiotics—did not follow from a rational application of Ehrlich's conception in the light of modern knowledge of the physiology of micro-organisms. Nevertheless, the study of the vital processes of the parasite and of the host seems to be the rational approach to the search for antimicrobial substances.

In this paper, the author discusses some of the aspects of microbial physiology and their bearing on the problem of microbial inhibition. These aspects are divided into five main groups : (1) the chemical anatomy of micro-organisms; (2) the physiology of micro-organisms; (3) the essential biosyntheses; (4) the biological superstructure; and (5) the microbial cell and its biological environment.

(1) The chemical components of the cell are considered from the point of view of its structure and functions, and the cardinal role of enzymes is discussed.

(2) The following formal categories of metabolic processes are reviewed : (*a*) the biosynthesis of protoplasm, (*b*) energy-yielding reactions, (*c*) " preparative " metabolic processes, and (*d*) the synthesis of characteristic metabolic products. Categories (*a*) and (*b*) are closely interlinked as series of essential metabolic reactions

RÉSUMÉ

Ehrlich avait émis l'idée que l'attaque d'un micro-organisme pathogène par un agent chimique devait avoir pour objectif de troubler de façon spécifique le métabolisme du parasite, sans nuire à l'organisme-hôte. Depuis lors, les connaissances sur les processus métaboliques se sont beaucoup développées, mais, il faut le reconnaître, dans certaines directions plus que dans d'autres. On peut relever aussi que la découverte de deux groupes importants de substances thérapeutiques antimicrobiennes — les sulfamides et les antibiotiques naturels — n'est pas le résultat de l'application logique des idées d'Ehrlich, interprétées à la lumière des notions modernes de physiologie microbienne. Cependant, l'étude des processus vitaux du parasite et de l'hôte semble être la voie la plus rationnelle à suivre dans la recherche de substances antimicrobiennes.

L'auteur discute, dans ce travail, certains aspects de la physiologie microbienne et leurs rapports avec le problème de l'inhibition de la croissance : 1) anatomie chimique des micro-organismes; 2) physiologie microbienne; 3) principales biosynthèses; 4) superstructure biologique; 5) cellule microbienne et conditions biologiques ambiantes.

1) Les composants chimiques de la cellule sont étudiés au point de vue structure et fonctions, et le rôle primordial des enzymes est souligné.

2) Les processus métaboliques suivants sont passés en revue : *a*) biosynthèse du protoplasme, *b*) réactions productrices d'énergie, *c*) processus métaboliques « préparatoires », *d*) synthèse de métabolites caractéristiques. Les catégories *a*) et *b*) sont étroitement liées, car elles représentent les réactions métaboliques néces-

necessary for the sheer increase of microbial protoplasm. Categories (*c*) and (*d*) may not be essential for the synthesis of protoplasm—at least for an organism growing in isolation in a nutritionally-suitable environment—but may be important for the life of a given organism in a biologically heterogeneous environment.

(3) The subjects dealt with in this group include : the synthesis of proteins and amino-acids; the components of enzyme systems concerned with the synthesizing and energy-yielding processes (*a*) and (*b*) above; the bearing of nutritional studies on the discovery of essential metabolic processes; the conception and biosynthesis of essential metabolites; the mode of action of sulfanilamide; and the rational design of antimetabolite growth-inhibitors and the successes and failures obtained with them.

(4) Among the subjects discussed in this group are : the relationship between the biological functions of the cell, such as nuclear division, cell-division, etc., and the essential metabolic processes necessary for protoplasmic increase; the possibility of interference with these biological functions; and the problem of the modes of action of the natural antibiotics.

(5) The differentiation between the effect of noxious agents on the parasite and on the host, and the special growth requirements of the viruses and of the so-called " pleuropneumonia-like " organisms, are considered.

saires à l'accroissement protoplasmique. Les facteurs *c*) et *d*) peuvent ne pas jouer un rôle essentiel dans la synthèse du protoplasme — du moins s'il s'agit d'un organisme croissant isolément, dans des conditions nutritionnelles favorables — mais elles peuvent être essentielles dans la vie d'un organisme se développant dans un milieu hétérogène.

3) Dans ce groupe sont traités les sujets suivants : synthèse des protéines et des amino-acides; composants des systèmes enzymatiques intervenant dans les processus de synthèse et de libération d'énergie, mentionnés sous *a*) et *b*); rapports entre les études relatives à la nutrition et la découverte de métabolites essentiels; mode d'action de la sulfanilamide; utilisation d'inhibiteurs de croissance choisis parce qu'étant des antimétabolites; succès et échecs de ces essais.

4) Parmi les questions discutées dans ce groupe figurent : la relation entre les fonctions biologiques de la cellule — division nucléaire ou cellulaire, etc. — et les processus métaboliques fondamentaux nécessaires à l'accroissement protoplasmique; les possibilités d'immixtion dans ces fonctions biologiques; le mode d'action des antibiotiques naturels.

5) L'effet des agents nocifs sur le parasite et sur l'hôte respectivement, ainsi que les conditions spéciales qu'exige la culture des virus et celle des *Borrelomyces* sont examinés.

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