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BIOCHEMICAL PRINCIPLES IN PHARMACY

ENZYMOLOGY: CHEMOTHERAPY: DISINFECTION: DEHYDRATION AND LIFE PROCESSES: CELL ARCHITECTURE

INTRODUCTION

I HAVE for some time now felt that I would like to point out to a pharmaceutical audience the important contribution made to the pharmaceutical sciences by the application of the principles of biochemistry. This contribution has been of rapidly increasing importance in the last few decades and I believe that its importance will increase even more rapidly in the future.

Biochemistry, as the name implies, is the chemistry of living matter, or the chemistry of the reactions occurring in living organisms. It would be quite impossible in the time at our disposal to discuss all the points at which biochemical principles are influencing pharmacy. Rather than attempt to cover the whole field in a superficial manner I have chosen to discuss, as examples, two principal biochemical topics. The first is the importance of enzymes, and the second the importance of water in life processes and especially the importance of the absence of water in the survival of life under adverse conditions; the latter since it affects the preparation of certain pharmaceutical products in a sterile condition.

One of the older difficulties of the biologist was to understand how plants and animals could, at temperatures below 40° C., bring about in their bodies reactions which, in the chemical laboratory, required high temperatures, high pressures and such powerful reagents as concentrated sulphuric acid and caustic alkali. The difficulty was solved by the discovery of the organic catalysts now called enzymes and known to be protein in nature. Enzymology is a fundamental branch of biochemistry and of pharmacology. Many enzyme systems necessary to the normal functioning of the animal body are inhibited, or less often activated, by substances used as drugs; indeed in a growing number of instances it is believed that the medicinal action of the drug is mediated by such inhibition or activation. Further, enzyme systems known to be essential to micro-organisms are usually inhibited, sometimes specifically, by substances used as disinfectants. The importance of a knowledge of enzymology to the pharmacist is thus obvious.

The transition from empiricism to a study based upon biochemical principles is nowhere more clearly seen than in the work concerned with

the discovery and evaluation of antiseptics and disinfectants. Originally, antiseptics were simply substances preventing sepsis in wounds. Disinfectants rendered safe disease-carrying materials such as sputum and fæces or clothing, water and food, etc., in that after disinfection they no longer transmitted disease. With the development of medical bacteriology it became clear that many diseases, the infectious and contagious, were caused by development and multiplication of micro-organisms within the tissues of the patient; the spread of such diseases is caused by the transfer of the causal organisms from diseased to healthy persons. It later became apparent that the prevention of such diseases was progressing along three lines.

(1) The study of antiseptics developed into chemotherapy—the search for and study of, such chemicals as would kill, without excessive damage to the infected host tissues, bacteria which had already gained access to the body.

(2) The study of disinfectants developed into the search for and evaluation of, bactericides and bacteriostatics—substances capable of destroying, or preventing the multiplication of, bacteria in materials which are to be introduced into, or applied to, the body.

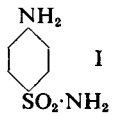
(3) Immunology which will not be considered further here.

Chemotherapy

Dursch[1] in 1785 was apparently the first to use the word chemotherapy but it was not until one hundred years later that the subject became important. Weigart[1] in 1873 studied the differential staining of tissues and bacteria and discussed his results with his cousin, Ehrlich. Ehrlich noted that methylene blue preferentially stained plasmodia in the blood stream at concentrations which left the blood corpuscles and other tissues unstained. In 1884 Gram[2] described a stain which was fixed by some bacteria but not by others. These observations gave rise to the idea that toxic dyes might be found which would kill invading bacteria but leave undamaged the tissue cells of the host. A few years later (1898–1900) Ehrlich[3] developed his "side-chain" or "receptor" theory concerning the mechanism of antigen-antibody reactions in serum involving haptophores which, it was suggested, anchor antibodies to the tissues and ergophores which were conceived to bring about chemical or physical alterations. This theory was easily extended to the chemotherapy of dyes and thence to other chemicals such as the organic arsenicals which possessed a more favourable "chemotherapeutic index"[4]. It was assumed that this index would be highest where parasitotropism (affinity for the invading organism) was much greater than organotropism (affinity for the host tissues). This assumption was later found to be invalid because the relative toxicity of the drug for parasite and host tissues outweighs simple affinity. Thus the concept of toxic groupings or toxophores was introduced. It led to such important, if now obsolescent remedies, as neoarsphenamine.

The idea of a close relationship between dyes and chemotherapeutic agents dominated the minds of chemists, particularly German chemists for over thirty years and led to the synthesis of such important drugs as

suramin, and mepacrine. Strangely enough it was the preparation (in 1932 by Meitsch and Klarer[5]) of a number of azo-dyes of sulphanilamide, shown to have effective antibacterial properties by Domagk[6], which led to the modern views of chemotherapeutic agents as analogues of normal metabolites. It was very soon realised[7] that the antibacterial activity of these substances was not due to their dye structure but to the simple *p*-aminobenzene sulphonamide grouping (I) which they contained. Woods[8] and Fildes[9] then made the most fertile suggestion that the *p*-amino-sulphonamide residue exerts its bacteriostatic action by replacing competitively the essential metabolite *p*-aminobenzoic acid (II) to which it is structurally related.



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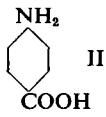

II

In confirmation it was shown that an excess of *p*-aminobenzoic acid could neutralise the bacteriostatic activity of several sulphonamides. Thus one molecule of *p*-aminobenzoic acid antagonises between 5000 and 25,000 molecules of sulphanilamide[9].

On the other hand, more recently, it has been suggested that sulphonamide bacteriostasis may be the result of enzyme inhibition. Sulphonamides certainly inhibit several enzyme systems concerned in essential metabolism. Indeed "integration of these effects shows that the tricarboxylic acid cycle, which constitutes one of the most important metabolic pools in the growth mechanism, is blocked by sulphonamides"[10]. In at least one case, i.e., pyruvate metabolism, *p*-aminobenzoic acid has been shown to antagonise the inhibition of the enzyme system caused by sulphathiazole. In some cases the inhibitory action of the sulphonamides may be antagonised by methylene blue or riboflavine suggesting that "the inhibitions and anti-inhibitions are independent of structural similarity"[10]. Which of these explanations of sulphonamide action is finally established as being correct is comparatively a minor matter. What is important is that two new lines of progress in chemotherapy have developed: (1) The elaboration and pharmacological testing of substances which are chemical analogues of essential metabolites, e.g., aminopterin which antagonises folic acid, and (2) the search for substances which inhibit essential enzyme systems of bacteria but which do not seriously affect adversely the enzyme systems of the host cells.

Disinfectants, Bacteriostatics and Bactericides

Probably the earliest reasonably quantitative attempts to compare and evaluate disinfectants were the experiments of Koch[11] in 1881. He dried anthrax spores on threads and dipped these into disinfectant solutions for various periods of time. After washing, the threads were transferred to fresh culture media. In this way it was possible to compare disinfectants and to ascertain the relation between the concentration of the disinfectant and the time required to sterilise the threads. But the

threads were difficult to wash and disinfectant was carried over into the fresh broth. Kronig and Paul[12] in 1897 replaced threads by more easily washed garnets. In 1903 Rideal and Walker[13] did away altogether with the solid supporting material. A suspension of a suitable organism (often *Bact. typhosus*) was added in known amounts to a solution of the disinfectant in predetermined concentration. At 2½ minute intervals disinfection was tested for by transfer of loopfuls of the infected solution to fresh broth, trusting to the dilution factor to prevent continuing action of the disinfectant during the test for growth. Phenol was used as the standard with which all other disinfectants were compared.

Five years later Chick and Martin[14] suggested making the test less artificial by causing the disinfectant to act in the presence of organic matter—fæces. Methods proposed by later workers have mainly been variations of these original techniques. Professor Berry[15] in his address from the Chair at the Pharmaceutical Conference at Harrogate in 1951 dealt fully with the uses, disadvantages and abuses of the Rideal-Walker type of test. At the beginning of this century scientific interest in radioactive breakdown was beginning and as a result of the work of Rutherford, Chick[16] in her original paper, was led to compare the rate of disinfection with the rate of a unimolecular reaction. This use of the word unimolecular was most unfortunate. The comparison has often been quoted and sometimes, in the past, with the implication that the disinfection reaction might be unimolecular. Chick herself was quite clear however, that the reaction could only be bimolecular (involving collision between antiseptic molecule and bacterium) and that first order kinetics were found only because of excess of one of the reactants (the antiseptic). In fact she established that the approximation to first order kinetics was quite superficial. Experiments with *Bact. paratyphosus* showed a departure from such kinetics in that the velocity diminished rapidly due to, it was suggested, "differences in resistance between individuals of various ages contained in such cultures". Further "When phenol is used as a disinfectant a logarithmic relation exists between its concentration and the time taken for disinfection. . . . (This). . . forms a marked contrast to the simple proportionality obtaining in the case of a chemical reaction of the unimolecular type". It should thus have been obvious from the first that approximations of only doubtful implication could result from attempts to assess a strictly biological response in terms of this form of kinetics which, logically as well as practically, is accurate only when applied to the rate of disintegration of a radioactive substance. Attempts were made to introduce into the first order equation a factor involving a power of the concentration so that a "true" velocity constant, independent of concentration, could be obtained. It was possible then to compare such constants for different disinfectants. But, since the power of the concentration varies from one disinfectant to another, it is doubtful whether such comparisons can provide a useful basis for the evaluation of disinfectants in general. Further, as discussed later, different classes of disinfectants differ in the way in which they kill bacteria. If phenol is used as a reference standard for a disinfectant of a different class acting by way of a different mechanism

then, as pointed out by Professor Berry[15], one of the most important principles of bioassay is broken.

A most thorough attempt to analyse and evaluate the overall action of disinfectants on bacteria was made by Berry and Michaels[17] who utilised extensively statistical and probit analyses originally devised by pharmacologists in order to obtain the greatest possible accuracy in bioassays. The work emphasised the complexity of the task. It might almost be said that each organism and each disinfectant requires separate treatment. In 1952 Eddy and Hinshelwood[18] summarised the position by writing—"Although the death of bacterial populations has been much studied no general agreement has been reached about the precise form of the curve relating the number of survivors to time or about the underlying processes which determine it". It has become obvious that the simple Rideal-Walker type of test, if carefully interpreted, is sufficient to ascertain the strength of disinfectant necessary in sanitary work. In problems connected more directly with surgery and the production of injections and other sterile pharmaceutical preparations, and especially at the academic level, the search for "a method" of "evaluating" disinfectants must be replaced by a study of the way in which different disinfectants affect the biochemical and biophysical processes of different micro-organisms.

This same conclusion may also be reached by a study of the results obtained by those workers whose original object was the study of the biochemistry of micro-organisms, and who went on to investigate the way in which normal metabolic processes may be modified by the addition of chemicals, which, in fact, are also used in higher concentrations as disinfectants. It appears that it is not just a question of the life or death of the organism. Small quantities of certain chemicals may modify, for example, the fermentative reactions by means of which an organism obtains energy while higher concentrations kill. A familiar example is the manufacture of glycerol by fermentation in the presence of sulphite, yet sulphites are widely used in the preservation of, for example, fruits. Mercuric salts inhibit sulphydryl enzymes and kill micro-organisms[19–22]. If the mercury is not present in too high a concentration both these processes may be reversed by sulphides or similar agents[23,24]. If the concentration of disinfectant is increased to such an extent that many metabolic pathways are interfered with, the organism dies. Different disinfectants first attack different metabolic processes.

Poole and Hinshelwood[25] suggested that some disinfectants interfere with the synthesis of metabolites essential for cell multiplication and prolong the lag phase of growth to such an extent that the organisms die before they multiply. Other disinfectants inhibit the rapid metabolic processes characteristic of the log phase while yet others interfere with the life processes of the mature organisms in the stationary phase. It is well known that organisms damaged, but not killed, by heat or disinfectants, when plated out and incubated, take longer, sometimes much longer, to form colonies[26]. Further, it is believed that different groups of disinfectants act in very different ways. To give only four examples of the ways in which disinfection may occur, phenolic substances coagulate

protein generally, and this of course includes enzymes. Certain heavy metals such as mercury inhibit sulphydryl enzymes. It is not certain how the surface active quaternary ammonium compounds act but one suggested mechanism is by alteration of cell permeability. Leakage of nitrogen and phosphorus-containing compounds from cells exposed to anionic and cationic surface active agents has been reported[27]. In the case of the acridine dyes it appears that germicidal activity depends upon their degree of ionisation and resides in the cation. It has been suggested that the acridine cation injures bacteria by competing with hydrogen ions for vital positions on dissociable acidic groups of respiratory enzymes[28]. The picture is still further complicated by the phenomena of adaptation and mutation. Micro-organisms are capable of adapting themselves to utilise, by fermentation, a substrate which at first they could not attack, i.e., they develop enzymes capable of breaking down the particular substrate. There has been much discussion about whether they do this as a result of stimulation by the new substrate or whether the substrate favours the survival of mutants which can ferment it. Pharmacists are well aware too that organisms grown in the presence of low concentrations of chemotherapeutic or antibiotic agents or disinfectants may become adapted to their environment by development of resistance.

Micro-organisms, when exposed to deleterious agents, especially heat, X- or β-rays or low concentrations of certain chemicals mutate giving rise to progeny lacking some, or equipped with different, enzyme systems. These agents are considered to act by destroying or altering the morphologically uncharacterised genes of the cell. Some geneticists have postulated the equivalence of one enzyme corresponding to one gene[29]. Often such mutated organisms survive, but heat, X- and β-rays in greater intensity cause disinfection. Little is known of the mechanism by which organisms are killed by such agents, but, once again, the action seems to be progressive. Kilner[30] reported that organisms killed by ultra-violet light could be reactivated by visible light. It has been postulated that absorption of one quantum of energy kills a bacterium provided that it hits a sensitive "target". Leu, Hains and Britscher[31] have calculated that an *E. coli* organism contains about 1000 such targets, of 8·6 mμ diameter, i.e., equivalent to the size of a molecule with a molecular weight of 2×10^5.

These ideas are reminiscent of the bimolecular theory of disinfection and Hinshelwood[32] has expressed his difficulty in believing that cell organisation is so dependent on localised structures that a single quantum energy or a single molecule of poison can possibly disrupt it. Those who would attribute a very simple mechanism to disinfection should bear in mind the fact that from a biochemical point of view the unicellular organisms, far from being simple, are more complex, possibly more highly evolved, than the cells of the higher animals. While in some cases the mechanism of disinfection may be simple, it is more likely in most cases to be complex. If complex, the disinfectant must be adsorbed or otherwise taken up by the bacterial surface; diffuse through the outer-membrane (possibly passing a lipid barrier); diffuse into the cellular

cytoplasm; react with some enzyme system progressively or instantaneously, reversibly or irreversibly. Possibly the organism may at first respond by using alternative metabolic pathways. Later, owing to increasing local concentration of disinfectant the disorganisation increases to such an extent that the capacity of the cell for division and growth is lost (bacteriostasis). Later, subsequent to inhibition of normal metabolism (and there is some evidence that anabolic processes are more inhibition-sensitive than catabolic processes) lytic reactions may supervene[33] and finally increase to such an extent that normal metabolism cannot be restored (bactericidal action).

It is small wonder that the time-survivor curves of disinfection processes are not straight lines! How could such a complex set of biochemical reactions be expected to give a straight line graph of any but the most misleading kind even after the application to the data of all the rites and ceremonies of orthodox statistics? Indeed, it would appear that attempts should be made to find experimental conditions which cause differences and deviations to be emphasised so that by further study the underlying biochemical complexities can be unravelled.

Pharmacological Activity Related to Enzyme and End-organ Structure

It is not only in the field of antibacterial action that enzymology has become important. As soon as it was realised that nerve impulses are transmitted across gaps whether in the nervous system itself or between nerve ending and muscle, gland or other end organ, by means of chemical substances the importance of biochemical considerations as a basis for the explanation of pharmacological activity became apparent. For peripheral sympathetic nerve endings the transmitter is noradrenaline or adrenaline, for the rest of the nervous system there is good evidence that the transmitter is acetylcholine and it is to this latter, as an example, and to the neuromuscular junction that these present remarks will be confined, although similar considerations are now thought to apply throughout the nervous system[34]. If an impulse is transmitted by a chemical substance the end organ will probably continue to be stimulated until the substance is destroyed. A new impulse can then be carried by freshly liberated substance. It has been established that acetylcholine exists in a bound, inactive form at nerve endings, that a nerve impulse liberates some free acetylcholine and that such is the local concentration of acetylcholinesterase that the liberated acetylcholine can be completely destroyed locally before the arrival of the next nerve impulse[35]. Acetylcholine has thus several properties. It "fits" the molecular surface of the muscle end-plates and initiates a contraction. It "fits" the surface of the acetylcholinesterase molecule and is fairly easily hydrolysed by it.

Any substance therefore which interferes with these activities may show resultant, characteristic, pharmacological activity thus:—(1) The substance may "fit" the active enzyme surface but be hydrolysed by it slowly or not at all, i.e., it may be a simple inhibitor of acetylcholinesterase. Such drugs, on injection, act in many ways like acetylcholine since by

inactivating the enzyme they preserve that substance when liberated locally at the nerve endings. Examples are eserine and dyflos (di-*iso*propylphosphorofluoridate, DFP) but whereas the inhibition produced by the former is reversible and the pharmacological action comparatively transient, dyflos causes irreversible inhibition and prolonged action and fresh enzyme must be produced by the body before normal physiological function is restored. (2) The substance may "fit" the end organ surface but not be hydrolysed so easily by the acetylcholinesterase. In this case two possibilities arise. (*a*) It may stimulate the end organ in the same way as does acetylcholine. In this case it will have an action similar to acetylcholine but of much longer duration; such a drug is carbachol. (*b*) It may fail to stimulate the end organ, but cover it in such a way as to prevent stimulation by acetylcholine. Should the end organ being considered be skeletal muscle, neuromuscular block will then result—the reverse effect of an injection of either a drug belonging to group (*a*) or of a drug with anticholinesterase activity.

It has been suggested that the different spacing of the active centres at neuromuscular junctions and synapses explains the differences in the actions of pentamethonium and decamethonium which have corresponding differences in carbon chain length[36]. It should be borne in mind that acetylcholine is a quaternary ammonium compound so that other members of this class are analogous. Since something is known of the structure and special relationships of the active centres on acetylcholinesterase, it may well be that a comparison of the anticholinesterase activity of these analogues with their relative neuromuscular blocking effects will throw light on the molecular structure of the parasympathetic receptors.

Local anæsthetics possess considerable anticholinesterase activity and at one time it looked as though this might be of importance in explaining their ability to block the conduction of sensory nerve impulses. The presence of acetylcholine and acetylcholinesterase within some nerve fibres as well as at the synapses has been demonstrated. It has been claimed[37] by some, although without evidence yet, that the action-current is propagated along the nerve fibre by liberation of acetylcholine, followed by its hydrolysis by acetylcholinesterase resulting in restoration of the resting potential. Local anæsthetics are anticholinesterases and it was tempting to think that they might act by inhibiting the acetylcholinesterase and so preventing the restoration of the resting potential.

Such a theory would involve the conclusion that all antiacetylcholinesterases should block nerve conduction when applied locally. It has been shown that this is true to some extent for eserine and dyflos[38]; the block, like the anticholinesterase inhibition, being reversible in the case of the former drug but irreversible in the case of the latter[39]. On the other hand, neostigmine, comparable to eserine in anticholinesterase activity, does not block nerve conduction. However, this has been shown to be due to the fact that neostigmine is a strong water soluble quaternary ammonium base which cannot penetrate the lipid nerve membranes while eserine, a tertiary amine, can[38].

There are, however, other difficulties. If acetylcholine is associated with a depolarised nerve membrane and local anæsthetics prevent the disappearance of acetylcholine then anæsthetised nerve membranes should presumably be depolarised. It has been shown that nerve membranes blocked by direct application of procaine solutions are not depolarised[40]. Further, when a considerable number of local anæsthetics were placed in order of increasing anæsthetic potency they were approximately in order of increasing ability to inhibit pseudocholinesterase of serum but not in order of increasing ability to inhibit acetylcholinesterase of brain tissue[41].

However, these difficulties may not be insuperable. The relations between potassium and sodium ions and acetylcholine and depolarisation and restoration of resting potential are by no means established and pseudocholinesterase as well as acetylcholinesterase regularly occurs in most parts of the nervous system although its function is as yet doubtful. It would appear that in future the development of drugs designed to act on the sensory nervous system, such as local anæsthetics, or neuromuscular blocking agents at skeletal neuromuscular junctions will be closely bound up with the study of the mechanism of the transmission of the nerve impulse and to a large extent will be linked with acetylcholinesterase inhibition or possibly activation. The same type of relationship has been found with other classes of drugs and enzyme-substrate systems. These developments may be generalised. The older methods of research in therapeutics consisted in testing, largely empirically, vegetable and animal extracts and isolated chemical principles for their therapeutic possibilities—often simply for their capacity to suppress symptoms. Later, synthetic chemicals and analogues of established drugs were examined in the same way. Attempts were made to establish relations between chemical constitution and pharmacological activity but exceptions to formulated rules were frequent and success in synthesising a new useful drug contained a large element of luck.

The new approach is quite different. Normal biochemical processes are studied and compared with the abnormal processes of disease particularly at the cellular and enzyme level. The next step is to find or synthesise substances, usually analogues of normal metabolites, intermediates, or enzyme substrates, which will replace, inhibit, side track or otherwise overcome the abnormal process or so activate the normal processes that recovery takes place. This is another way in which therapeutics is passing from the realms of empiricism to those of the sciences.

Pharmaceutical Enzyme Preparations

Before leaving this subject of enzymology I should like to refer to the introduction of certain preparations of digestive enzymes well known to pharmacists. As far back as 1783 Spallanzani[42] demonstrated the liquefaction of meat by the gastric juice of hawks. In 1836 Schwann described and gave the name pepsin to the proteolytic enzyme of gastric juice. Pepsin, in the crude form of the dried stomach lining was introduced in the 1874 "Additions" to the British Pharmacopœia of 1867.

The action of pancreatic juice on albumin was observed in 1836 by Purkinje and Papenheim. In 1856 Corvisart described trypsin while in 1862 Danilewsky separated pancreatic amylase from trypsin. A preparation of pancreatic enzymes known as Pancreatic Solution became official in the British Pharmacopœia 1898.

Vegetable digestive enzymes were also discovered about the same time. It is impossible to say how long it has been known that the juice of *Carica papaya* and other species of *Carica* has an energetic action on meat. Possibly the earliest scientific account was given by Griffith Hughes in 1750, followed by that of Browne in 1756. The enzyme preparation was first studied by Willmach in 1878 and the name Papain given by Wurtz in 1879. Although it was never included in a British Pharmacopœia it was given a monograph in the first British Pharmaceutical Codex in 1907 and has had extensive trials in the cleansing of wounds and burns and has also been used in dentistry[43]. Pepsin and pancreatin (which has replaced the solution) still remain in the British Pharmacopœia and papain in the Codex. Malt too has been known from antiquity but it was in 1833 that Payen and Persoz separated active amylase from it.

It was thus towards the end of the nineteenth century that the digestive processes were described in terms of enzyme action in a scientific manner, the specific enzymes being separated and studied. This led not only to the introduction of pepsin and pancreatin and their vegetable counterparts, papain and maltase, but also to the malted and predigested foods still widely used in digestive disorders and for feeding infants and invalids. It is possible that the historical significance of these researches on digestive enzymes has been overlooked. The treatment of faulty digestion by use of pepsin or pancreatin is as much an example of modern replacement therapy as the alleviation of diabetes by injection of insulin. Conference papers relating to pepsin[44], pancreatin[45,46] and papain[47] have been communicated even in recent years.

Finally, one must mention the more recently introduced and important blood preparations including the official enzyme Human Thrombin. In addition, several chemical substances are now administered to shorten or lengthen the clotting time of blood so that the interest to a pharmacist of the biochemistry of blood is obvious.

Use of Enzymes in Analysis

The use of enzyme inhibition as a sensitive analytical tool has been suggested. Cholinesterase is inhibited by eserine in concentrations as low as 10^{-7} molar and this reaction has been used to estimate eserine in galenical preparations[48] and for the detection and estimation of the alkaloid in toxicological work[49]. Anticholinesterase drugs are used in medicine in very low concentration. For example, the 1 ml. ampoules of Injection of Neostigmine Methylsulphate contain only 0·5 mg. of neostigmine. In a paper to be read at this Conference it will be shown that there is more than enough neostigmine in a single ampoule for its concentration to be estimated and with less than a 10 per cent. error, by means of cholinesterase inhibition. Similar methods will identify, and estimate

mustine hydrochloride. The need for such methods is obvious when the present B.P.C. monograph is considered.

Survival of Enzymes and Micro-organisms in Systems of Low Moisture Content

I must confess that I have always been fascinated by the fact that if one of several of the properties of water had been only slightly different, life as we know it would have been impossible. If the maximum density of water had happened to be below 0° C. instead of at + 4° C. ponds and seas would have frozen from the bottom upwards. There would have been no thin layer of ice on the top to protect fish and other aquatic forms of life, from the effects of the cold of winter weather and the ice ages. It is improbable that the deep cold ice would have thawed during the summer. Alterations, not very great, in the vapour pressure and hence in the boiling point and rate of evaporation of water would have rendered impossible anything approaching our present climate with the beneficent circulation of water from the seas to the atmosphere and on to the land. One could go on to mention such properties as the dissociation constant (giving neutrality in the region of pH 7·0) and the dielectric constant (controlling the degree of ionisation of electrolytes). Further, using modern terminology, one might say that if hydrogen bonding of water molecules had not occurred evolution must of necessity have had a very different pattern and one wonders if man could have resulted. Having often amused myself by speculation along such lines, I was naturally most interested in the work of Baker[50] (1902 onwards) demonstrating the importance of water as a catalyst in inorganic chemistry. Sulphur and phosphorus may be distilled unchanged in oxygen in the entire absence of moisture. Baker showed further that oxygen and hydrogen combine only slowly under such conditions, no explosion occurring even in the presence of a spiral of silver wire heated almost to melting point[50].

It was these considerations which caused me to want to study the behaviour, especially the survival or nonsurvival, of enzymes and micro-organisms in systems of low moisture content. It was fortunate for me that pharmacists are interested in the sterilisation of two such systems, namely powders and oils so that it was possible to work in such a way that the results might be useful as well as being of academic interest.

Moisture and Heat

In some preliminary work, it was found possible to heat pepsin powder for one hour at 120° C. without appreciable loss of activity. Later, working with lipase powder[51] it was shown that the amount of moisture present in the powder during heating was a crucial factor for the survival of enzyme activity. Heating for one hour at 110° C. left the activity unchanged, if the powder had previously been dried over P_2O_5, but resulted in the loss of all activity in the case of a damp, but still free flowing powder heated in a closed container. If heated in an open dish the moisture rapidly escaped from the powder and only a portion of the activity was lost. If the powder were suspended in oil before heating,

moisture was still an important factor but for a given moisture content of the powder the loss in enzyme activity was less than if the powder had been heated in a closed vessel but more than if it had been heated in an open dish. Possibly some of the water diffused into the oil. Drying renders enzymes more stable not only to heat but also to other agents. Moist acetylcholinesterase is destroyed by treatment with acetone or ether[52] but dried brain tissue may be repeatedly extracted with either acetone or ether without loss of acetylcholinesterase activity[53].

In experiments in which a spray drier was used to produce the powders, it was shown that many delicate enzyme systems and easily oxidised substances such as adrenaline and ascorbic acid could be spray-dried in a current of air having an initial temperature of up to 120° C. without loss of activity[54]. The preservation of these delicate substances was attributed to very rapid drying and subsequent stability in the absence of moisture.

Preservation by drying applies not only to enzymes but also to micro-organisms. It has of course been known for a long time that foods and drugs can be preserved by drying but it is rather curious that drying should also preserve the micro-organisms which are mainly responsible for spoilage in such products. It has long been known too that spores may remain alive for long periods of time in such powders as dust or dry earth. More recently the preservation of bacterial cultures by freeze-drying has made familiar to us the idea of conserving even the more delicate types of vegetative bacteria for considerable periods of time in the dry state. The freeze drying process[55] usually results in some destruction which may amount to over 90 per cent. of the total population but once dried the organisms are comparatively stable[56,57].

With regard to the heat resistance of micro-organisms in the dry state, Cameron[58] showed that many bacterial species when desiccated will resist temperatures of over 100° C. for 10–60 minutes; while spores may withstand a temperature of 130° C. for over three hours. Topley and Wilson[59] have suggested that the heat resistance of spores may be due to their low total moisture content. It has been shown by Leiveth[60] that the temperature coagulation of protein, e.g., dried egg albumin, is related to the moisture content.

By a process of spray drying[61], powders were obtained containing a known number of micro-organisms (usually vegetative bacteria or spores) in even distribution[62]. It was possible to investigate quantitatively the effect of moisture on the death-rate of organisms in such powders when exposed to either high temperatures or bactericidal chemical agents. The following conclusions were established: (1) Vegetative organisms (*Bact. lactis aerogenes* in peptone powders) are more susceptible to heat destruction than is the enzyme lipase. They are, however, much more resistant to heat in the dry rather than in the wet state. Whereas 50 per cent. of the bacteria were killed in thirty seconds at 55° C. in liquid suspension it took about forty minutes at 70° C. in the dry state to produce the same mortality. (2) The thermal resistance of spores in powders resembles the thermal resistance of enzymes in dry powders. (3) Even in the dry state vegetative bacteria are much more susceptible to heat than

are spores; one hour at 110° C. destroying all the former, while one hour at 140° C. was necessary to destroy all the latter. (4) As with enzymes vegetative bacteria were more easily killed by heat in moisture containing powders than when quite dry. 7·2 per cent. moisture lowered the temperature for killing in one hour from 110° C. to 90° C. provided the heating was carried out in a closed container. In an open container the majority of the water was removed so quickly that it had little effect. (5) Some unexpected results were obtained with *B. subtilis* spores. One hour at 140° C. was required for sterilisation whether the powder was dry or contained 7·3 per cent. moisture and whether the containers were open or closed. This result may have something to do with the impervious nature of spore coats. Heat susceptibility may well be influenced by the moisture content of the bacterial cell[63] rather than by that of the ambient medium.

The Influence of Moisture on the Action of Disinfectants in Powders

In earlier work relating to the action of disinfectants on dried micro-organisms the latter were obtained from samples of either dust or dried soil; or by dipping threads, coverslips, garnets, etc., into cultures of known organsisms and allowing the adherent film to dry. In the first experiments the infection was mixed and consisted of uncharacterised and usually unknown species although the powders might be so well mixed that the organisms were evenly distributed. Obviously any experiments described could never be repeated exactly since the next sample of dust or soil might contain quite different organisms of quite different resistance. In the later experiments known organisms were used but the distribution was uneven and the method could yield only, at best, approximate results. In all experiments there had, no doubt, been extensive but unascertained death of organisms during the drying process so that it was a selected sample of viable organisms which was stored, heated or exposed to disinfectants. Later the much superior method of freeze-drying bacterial cultures was introduced and it was established that even vegetative bacteria may survive in the dry state for many years, especially in evacuated tubes[64]. However, not much work was done on the rate at which they die[56,65,66]. After drying, the immediate death rate was often high. The proportion of organisms viable after some months might be less than 1 per cent. of those originally present in the suspension.

Some of the difficulties associated with this work were overcome by the use of the spray-dried powders described above[62,63]. By spray drying, *Bact. lactis aerogenes* was killed to the extent of 98 per cent. under the conditions most favourable for survival, and the resultant powder had to be milled before an even distribution of the bateria was obtained. The same was true of *Str. fæcalis* if high temperatures (air inlet to the drier 180° C.) were used but with a lower inlet temperature, under the most favourable conditions, over 85 per cent. of the bacteria survived the drying processes and were found to be evenly distributed in the resultant powder[67].

Using peptone as the supporting material, the viable count of the dried powder fell rapidly, with *Bact. lactis aerogenes* from 542,600 to 6400 in

thirty seven days, but with *Str. fæcalis* starting with 10,000,000 per g. it was possible to obtain powders containing 3,000,000 organisms per g. after one week, 40,000 after a month and 2000 after six months. Although there is a considerable death rate in such powders, it is possible to use them to investigate the extent to which disinfectants increase the rate of kill. Experiments with the spores of *B. subtilis* proved to be relatively easy since over 95 per cent. of the spores survived spray-drying and the survivors proved to be evenly distributed in the resultant powders. At the commencement of the spray-drying process the organisms were usually suspended in a relatively dilute medium of 2–10 per cent. of total solids content and of known hydrogen ion concentration. At the end of the process less than 5 per cent. of moisture was present. During the process, therefore, there must have been a considerable rise in osmotic pressure, especially in the presence of salts, while it is difficult to forecast what happens to the hydrogen ion concentration or indeed to understand its significance in concentrated solutions or pastes.

Micro-organisms are very sensitive to the nature of the medium in which they happen to be and it was therefore of interest to dry the spores of *B. subtilis* on a variety of salts, using high air-inlet temperatures[68]. Acid and alkaline salts were used and it was known that in some cases at the high air-inlet temperatures anhydrous salts resulted[69]. The spores were remarkably resistant. 50 per cent. survived drying on sodium chloride and more than 30 per cent. survived drying on sodium carbonate. Phosphates seemed almost to have a conservative action. On sodium acid phosphate, more than 95 per cent. survived, so that this salt had no more harmful effect than peptone. Even such chemically active salts as sodium nitrite, potassium chlorate and calcium formate did not result in sterility, 2, 22 and 26 per cent. respectively of the spores surviving the drying process. At first it had appeared that it might be possible to sterilise materials by introducing relatively small quantities of disinfectants into the solutions before spray-drying. During the drying process the concentration of non-volatile disinfectants should increase about ten times, unless limited by insolubility of the disinfectant. The increased temperature in the drier should increase the death rate of the bacteria. It was even thought that by using a volatile disinfectant the resultant sterile powder might be free from, or contain only a trace of disinfectant. However, the non-destructive effects of the spray-drying process operated against the desired result. Spray-drying spore suspensions in solutions containing 0·5 per cent. phenol, 0·2 per cent. chlorocresol, 0·002 per cent. phenyl mercuric nitrate, or 0·5 per cent. resorcinol in all cases resulted in less than 30 per cent. mortality[68].

By combining the usual bactericides with salt solutions, surface active agents and high air-inlet temperatures, the percentage mortality could be increased but not above 90 per cent. Sterility was obtained by drying the *B. subtilis* spores in peptone in the presence of 0·4 per cent. formaldehyde but this is not surprising since formaldehyde is a volatile bactericide which may be used to sterilise dry powders[70].

These results strikingly confirmed the non-destructive effects of the

spray-drying process. Not only is there little temperature destruction of spores even with air inlet temperatures up to 190° C. but the drying is so rapid that any disinfectant present has no time to be effective even though its concentration is rapidly increasing. In the absence of moisture, disinfectants are practically inert unless volatile.

Having obtained powders containing known numbers of named vegetative bacteria or bacterial spores, it became possible to ascertain the effects of moisture content on the viability of the organisms in such powders on storage. *B. subtilis* spores in powders sufficiently dry to be free flowing remained viable practically indefinitely. This raised an interesting consideration. In dilute aqueous peptone the spores germinate and the resultant vegetative bacteria rapidly multiply yet we know that the process of drying vegetative bacteria, unless by freeze-drying or spray-drying, under the most favourable conditions, kills a large proportion. It appeared likely therefore that there would be a critical moisture content of the powders (or concentration of peptone solution) at which the spores would germinate but the resultant bacteria would find the conditions unfavourable for growth. There were in fact found to be two critical moisture contents (1) a moisture content (50 per cent. for peptone powders, 10 per cent. for lactose powders), below which the spores did not germinate but remained viable and resistant. (2) A very much higher water content at which germination and multiplication of the vegetative forms took place in the presence of nutrient (e.g., peptone powders). The interesting point was that between these two critical moisture contents, the spores germinated, but far from multiplying, the resultant vegetative forms died. Indeed, at one time we had hoped to be able to sterilise powders by increasing the moisture content sufficiently for the spores to germinate and die and then simply redrying the powders. Unfortunately the critical moisture content required for this always resulted in the powders becoming pastes which introduced technical difficulties in redrying[71].

The Influence of Moisture on the Action of Disinfectants in Oils

Oils and fats constitute systems of low moisture content in which bacteria may occur. It was found that these systems could be contaminated by stirring in infected powders[72]. The bacteria might be surrounded by a layer of dried medium such as peptone and so not be in direct contact with the oil or fat. In some experiments this objection was overcome by spraying a bacterial suspension in an acetone solution of stearin. The resultant infected stearin was mixed with oils or fats to give the required suspension. Three conclusions emerged from the work using contaminated oils. (1) Spores (*B. subtilis*) may remain viable and resistant in oils for long periods of time (over two years). This is true even when the spores have been freed from surrounding solid nutrient media. (2) Vegetative organisms (*Str. fœcalis*) die in oils at about the same rate as in the powder used to infect the oil. The oil does not increase the death rate and the experiment can be so arranged that after six months' storage the oil may still contain 100,000 organisms per g. (3) Introducing disinfectants even in high concentration, for example, 2 per cent. chlorocresol, into the oil

suspension has little, if any, effect on the viability of spores or vegetative organisms.

It is of pharmaceutical interest to note that although spores remain viable in powders and oils for long periods of time the degree of contamination of powders and oils was never found to increase on storage.

Summarising, it can be stated that water plays an essential part in the destruction of micro-organisms either by (1) chemical agents (bactericides), or (2) heat below the temperatures at which organic decomposition occurs.

Cell Architecture

When considering the long periods of time during which dried bacterial spores can remain viable as shown by their capacity to germinate under favourable conditions, one is tempted to reflect on the nature of life. It has been said that "Solid matter is not compatible with life". Yet bacterial spores dried and stored over P_2O_5 seem to be very much of the nature of "solid matter". In what way are they "living"? They seem to remain as it were in suspended animation. If the synthetic and metabolic processes are suspended so are those of lysis and dissolution. Although I am emphasising the importance of water, I certainly would not care to go all the way with Goethe when he says in Faust:—

"Alles ist aus dem Wasser entsprungen
Alles wird durch das wasser erhalten."

In what ways then do dry "living" spores differ from, say, the "dead" powder which can be obtained by grinding such spores in a mill? The living spores have an internal structure or organisation; in the dead powder that structure has been destroyed although the units remain since many of the original enzyme activities persist. For a long time the importance of structure in protoplasm and cellular organisation has been realised and studied. Cytologists have established the importance of such structures as the nucleus and the chromosomes as well as mitochondria, Golgi apparatus, centrosomes, and microsomes. Recently it has become possible to associate specific enzymes with certain of these organelles. The possibility of the formation of particular enzymes in the cell has been linked to specific genes and in some cases the ratio of one enzyme for one gene has been suggested. Genes are certainly associated with chromosomes. At one time the biochemist was accused, perhaps with some justification, of treating the cell as a "bag of enzymes". This is no longer the case. It is becoming increasingly probable that the arrangement of the enzymes in the cell is important. They must act on substrates in a given order and to do this must occupy a particular location, relative to each other in space, i.e., a cellular architecture is necessary. Here then is a possible explanation of the difference between dried living cells and the powder produced by grinding them. The latter contains all the biochemical units but it is only when these have the requisite space relationships that life (i.e., the possibility of growth and reproduction) is present. It may be that in the future life will be characterised in terms of biochemical architecture.

The recent advances in this subject have been made possible by the development of three techniques (1) electron microscopy; (2) submicrochemical reactions applied to histological preparations whereby chemicals and enzymes may be located in individual cells or even in specific parts of cells; (3) cell fractionation involving the partial disruption of cells followed by separation by high speed centrifugation of various constituents or organelles such as nuclei, mitochondria, chromosomes and, of course, supernatant clear cytoplasm or hyaloplasm. The various fractions are then examined separately for chemical composition and enzyme content. None of the three methods is beyond criticism but when the results of two or three methods are confirmatory the conclusion has at least a high degree of probability. In this way it has been shown that certain enzymes are associated specifically with certain of the morphological structures.

In the short time at our disposal it is impossible to review the now extensive literature relating to this subject; one or two examples must suffice. It has been found that cytochrome oxidase is located exclusively in the mitochondria of certain cells[73] and the same location for succinic dehydrogenase has been established[74]. On the other hand, glycolytic enzymes have been found in cell nuclei isolated by three different procedures and it seems reasonable to suppose that these enzymes are true nuclear constituents. Clearly the normal metabolic cycles take place in morphologically different structures of the cells. It would appear likely that the substrates, intermediates, and end-products follow definite pathways within the cells. Such an idea is reminiscent of the way in which the food vacuoles in paramecium are carried by a streaming of the ectoplasm along a predetermined route within this monocellular organism.

Sometimes enzymes are associated with the surfaces of cells. Acetylcholinesterase has been found to be concentrated in the sheath of the giant fibre of the squid[75], practically none of the enzyme being found in the axioplasm. This observation may be connected with the conduction of nerve action currents along the surface of the fibres. It has been suggested that the occurrence of phosphatases at the surface of cells may be necessary to hydrolyse organic phosphates so that the organic fragment can be absorbed and metabolised. It has been shown that alkaline phosphatase occurs in high concentration in the nuclear membrane of certain cells[76]. Whether this enzyme plays a role in the transfer of metabolites from cytoplasm to nucleus or in the reverse direction is not yet established. Not only have cells an internal morphological structure but it is increasingly evident that the organelles themselves have a complex architecture. This has long been accepted in the case of the nucleus. There is now increasing evidence that the morphological similarities of the mitochondria of different tissues are paralleled by similarities in biochemical properties. It has been further found that inhibition of oxidative phosphorylation may be accompanied by a change of form of the mitochondria from rod like to spherical by swelling[77]. The complexity of the mitochondria has been summarised by Hogeboom and Schneider[78] as follows: "The general picture is that of an osmotically active system, protected from its environment by a relatively impermeable membrane, and containing a high

concentration of proteins (including enzymes) and metabolites in a diffusible state. In addition, a number of enzymes appear to be firmly bound to the structural framework of the mitochondrion".

It is not only in the cell organelles that structure is to be found. The electron microscope has disclosed structure in the optically empty hyaloplasm. A very fine reticular network is revealed, considered by some to be lamellar in form, by others to consist of vesicles and canaliculi. Much of this was forecast by Quastel and Wooldridge in 1927[79] who wrote "This does not imply, of course, that only the histological structures are involved: the smaller colloidal aggregates are just as much a part of the architecture of the organism". The same idea has recently been expressed in more modern terms by Schmitt as follows. The temporarily and spatially ordered coupling of energy upon the macromolecular lattices of protoplasm is at the core of most processes of cellular biology[80]. It is with elemental units having definite and functional orientation in space that the molecules of drugs must combine. These considerations suggest a firm basis for work relating pharmacological activity to the conformational structure of the drugs and of that portion of the cell architecture, the functions of which they alter. It is interesting to note that papers on this subject are beginning to appear in pharmaceutical literature[81,82].

It is true that the morphology of the bacterial cell differs from that of liver cells with which much of the described cytochemical work has been carried out but bacterial cells have a very definite morphology, and, since their respiratory and fermentation cycles are similar and indeed rather more complex, varied and self sufficing than those of the cells of the higher organisms, it is unlikely that there is not in the bacteria also a close linkage between structure and normal metabolism. Indeed such enzymes as succinic dehydrogenase, various cytochromes and Kreb's-cycle enzymes have been found to be associated with certain cytoplasmic granules 100–200Å. in diameter[83].

In conclusion, I want to make it clear that I do not think that in enzymology, cytochemistry or indeed in biochemistry we have or can have all the answers to all the questions. Nor am I one of those who think that we are near to the point where life can be created or entirely explained or even described in terms of chemistry and physics. I do think that we are at the beginning of a series of investigations and researches into the relation between structure and the vital processes of cells and cell-parts which will prove to be most fascinating and illuminating. Certainly there is a great future for fundamental researches concerning the biochemical aspects of the pharmaceutical sciences. This does not, of course, mean that we should neglect to consider such applied aspects as the discovery of better bactericides, chemotherapeutic agents and drugs with improved pharmacological actions. Indeed, on the one hand, the pursuit of such practical objectives is revealing much information of fundamental interest while, on the other hand, the fundamental research work is supplying a basis for the direction of technological investigations. The practical objectives will be more directly and easily attainable when we know more of the

underlying causes and mechanisms of biological processes. Here, as in so many other spheres of life, how true are words of Virgil when he says:—

"Felix qui potuit rerum cognoscere causas".

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