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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.

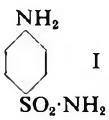
(3) Immunology which will not be considered further here.

## 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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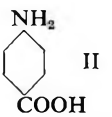


II

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 $2\frac{1}{2}$ 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 $\beta$-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 $\beta$-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 m$\mu$ diameter, i.e., equivalent to the size of a molecule with a molecular weight of $2 \times 10^5$.

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. fæcalis*) 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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# SCIENCE PAPERS AND DISCUSSIONS

## RESISTANCE OF CRYSTALLINE SUBSTANCES TO GAS STERILISATION

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THE manufacture of thermolabile drugs in a sterile form has always presented a difficult technical problem, requiring special apparatus and procedures. The maintenance of aseptic areas, the training and constant supervision of staff, the rigorous application at every stage of bacteriological testing, are examples of these problems, and may result in the rejection of batches because of contamination and their return for reprocessing or abandonment. The introduction of a "cold" sterilising process would, therefore, be a valuable addition to present aseptic techniques, and published reports on various bactericidal gases have suggested that such treatments have been successful. During the course of investigations on sterilisation by ultra-violet radiation we observed that on some occasions crystallised materials which had been contaminated with a liquid suspension of bacterial organisms and subsequently dried were more difficult to sterilise than materials contaminated with dry micro-organisms. As the majority of thermolabile drugs manufactured for pharmaceutical use are of a crystalline nature, it was thought that an investigation of this difference might have practical significance when applied to gas sterilisation. In general, it was found that materials crystallised from a bacteriologically contaminated liquor were more resistant to a gas exposure technique than similar materials contaminated with a bacterial "dust". This suggested the possibility of the inclusion of organisms within substances crystallised from such liquors, and so escaping contact with the sterilising gas. The phenomenon of included foreign matter in crystals is well known. Bentivoglio observed the inclusion of "rows of minute cavities filled with solution" in her work on the rate of growth of magnesium ammonium sulphate crystals[1] and similar observations were also made by Bunn[2]. There is, however, no reference in the literature to suggest that organisms may be so included and remain viable, with the exception of a report by Seriakowski[3] which mentions the survival of viable organisms in crystals of a phosphate growing in broth cultures. The experiments recorded here were designed to show that viable spores can survive within crystals and that such inclusion affects the sterilising ability of gaseous agents by preventing contact between organisms and agent.

### EXPERIMENTAL

An initial series of experiments was planned to establish methods for preparation, contamination and sterilisation of crystals. As a crystalline

material, Rochelle salt was chosen. It is readily soluble, easily crystallised, non-hygroscopic and has no action on the proteins in the broth or agar media used. The method of gas sterilisation described in the work by Bullock and Rawlins[4] in which the action of formaldehyde and air on *B. subtilis* spores was investigated in detail, appeared to be suitable for our purpose, and with modifications was adopted.

*Comparisons of Resistance to Sterilisation*

*Preparation of surface-contaminated crystals.* A solution of two parts of Rochelle salt in one part of water was autoclaved, cooled slightly, and then poured into a covered Pyrex dish and allowed to crystallise. Crystals of suitable size were then transferred to filter paper to dry; these were selected for regularity in size and shape, and freedom from adhering small crystals and aggregates. Tests for the presence of viable bacteria were made on a 10 per cent. representative sample of the number produced at each crystallisation. Each crystal was dissolved in 10 ml. of Bacto Difco nutrient broth and incubated at 30° C. for 17 hours. If no growth occurred, the broth was subcultured into a similar broth and incubated for the same time and at the same temperature. If, again, no growth occurred, the original broth was then inoculated with a suspension of *B. subtilis* spores to check that the lack of growth was not due to bacteriostasis. It was established that crystals grown and tested in this way did not show the presence of viable organisms on any occasion. The test organism used for contamination was *B. subtilis* (NCTC 3110). The organism was grown for 48 hours at 37° C. on modified agar slopes in Roux bottles and spores were collected by washing from the surface with sterile water and glass beads. After washing and centrifuging the spores were suspended in water. These suspensions were heated at 60° C. for 30 minutes, to kill vegetative organisms, and after examining microscopically, were filled into ampoules and freeze dried. The number of viable organisms in the resulting powder was approximately $10^8$/g. This was estimated by preparing suitable dilutions in agar pour plates using a layering technique to prevent surface spread as demonstrated to us by Mr. G. E. Davies. This powder was used to contaminate the exterior surfaces of the crystals previously produced. Sufficient spore powder to give a concentration of about $10^6$/g. was added to a weighed number of crystals in a bottle, and the bottle was then placed on a roller mill for one hour. A 10 per cent. representative sample of the crystals from each batch produced was tested bacteriologically to ensure that the desired contamination had taken place.

*Sterilisation of Surface-contaminated Crystals by Formaldehyde*

*Apparatus.* The apparatus resembled that of Bullock and Rawlins[4] (Fig. 1). Two grade 2 sintered glass filters (C.1 and C.2) were connected to a Drechsel bottle containing glass beads (A) and a bubbler with a fine jet (B), both containing solution of formaldehyde A.R., and air filtered through a cotton wool plug, was passed through at controlled rates, *via* a gas flow-meter (E), by pressure or vacuum. To estimate the

concentration of formaldehyde in the air passing through the apparatus, a sintered glass bubbler (D) containing sodium metabisulphite solution was switched into the system when desired, and removed when a measured volume of formaldehyde and air mixture had been scrubbed. A second bubbler was found to be unnecessary.

*Estimation of formaldehyde.* The concentration of formaldehyde in the air was estimated by the method of Goldman and Yagoda[5]. Four litres of formaldehyde and air were scrubbed by means of the bubbler containing 100 ml. of 1 per cent. w/v sodium metabisulphite solution, when the formaldehyde formed a non-volatile complex with the bisulphite. After removal of the bubbler from the system, 10 ml. of bisulphite solution was placed in a 250 ml. glass-stoppered flask and titrated to a dark blue end-point with 0·1N iodine, using starch indicator. Excess iodine was then destroyed by addition of 0·05N thiosulphate, added dropwise from a burette, and 0·01N solution of iodine then added to give a faint blue end-point. Twenty-five ml. of a solution of sodium carbonate 80 g., glacial acetic acid 20 ml., and water 500 ml., was then added to decompose the bisulphite-formaldehyde complex and the liberated bisulphite titrated with 0·01N iodine to a faint blue end-point. (Each ml. of 0·01N iodine is equivalent to 0·15 mg. of H·CHO.)

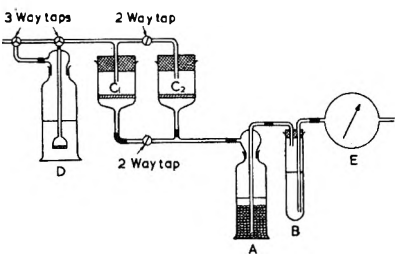


FIG. 1. Apparatus for subjecting the crystals to formaldehyde/air.

*Method.* It was shown in preliminary tests that with a flow rate of up to 800 ml. of air per minute through the apparatus, the concentration of formaldehyde in the air was about 2·4 mg. per litre (varying from 1·9–2·6 mg./litre). Contaminated crystals were exposed to these conditions and tested for the presence of viable spores by solution in broth and incubation as before. When no growth took place, inoculation with a *B. subtilis* spore suspension followed by incubation, produced growth, thus showing the absence of bacteriostasis due to residual formaldehyde. After five hours' exposure the crystals were sterile, and a six-hour exposure period was therefore used in subsequent experiments as a "standard sterilisation time".

*Isolation of non-sterile crystals from solution.* Sterile solutions of Rochelle salt were prepared and cooled to about 50° C. Diluted spore suspensions, prepared by suspending *B. subtilis* spores (freeze dried) in water, were added to give concentrations of $10^6$/g. of solution. The solutions were then allowed to crystallise under aseptic conditions, crystals of about the same size as the sterile crystals previously prepared being removed and dried as before. To ensure that there was contamination of the crystals as distinct from the mother liquor, the individual crystals from weighed groups of ten were dissolved in sterile water, and suitable dilutions prepared in agar pour plates. It was found that the

contamination varied between 400–700 organisms/g. of crystal. Crystals prepared in this way are described as "internally contaminated".

*Attempted sterilisation of internally contaminated crystals.* Similar weights of internally contaminated crystals and externally contaminated crystals (as control) were placed in the two gas chambers, $C_1$ and $C_2$. The air supply or vacuum was adjusted to give the required flow rate and the formaldehyde concentration in the air passing through the apparatus was measured. During the exposure of the crystals the chambers were shaken frequently to expose all crystal surfaces. At the end of the "standard sterilisation time" of 6 hours, ten crystals were taken from each chamber, and tested by dissolving in broth and incubating. A series of eleven experiments with five batches of crystals were thus performed to enable a qualitative indication to be made of the possibility of bacterial spores surviving inside crystals and the results are shown in Table I.

TABLE I

RESISTANCE OF CRYSTALS TO STERILISATION

| | |
|---|---|
| Number of crystals of each kind tested .. .. .. .. .. | 276 |
| External contamination: proportion of crystals found contaminated .. | 5/276 (1·8 per cent.) |
| Internal contamination: proportion of crystals found contaminated .. | 249/276 (90·2 per cent.) |

This result shows a difference between the ability of formaldehyde to sterilise crystals merely surface contaminated, and those crystallised from a contaminated liquor. Although the contamination in the former is higher, the period for which both lots of crystals were exposed was sufficient to sterilise in all but 1·8 per cent. The suggestion is made that the spores in the latter instance are protected from contact with the gas by inclusion within the crystal mass. In order to obtain quantitative estimates of the extent to which such inclusion occurs, another experiment, using groups of graded crystals, was made. Ten crystals of each kind in different size ranges were exposed in the two chambers to the gas procedure as before. The crystals were then removed and plated in nutrient agar. Tests had shown previously that the presence of residual formaldehyde on the Rochelle salt did not inhibit colony development and there was no advantage in prolonging incubation time beyond 17 hours. Table II shows results obtained from the treatment of representative groups in each size range.

TABLE II

VIABLE SPORES AFTER EXPOSURE OF GROUPS OF 20 CONTAMINATED CRYSTALS TO FORMALDEHYDE GAS

| Average weight of ten crystals g. | Contaminated externally | | Grown in contaminated solution | |
|---|---|---|---|---|
| | Spores/g. before gas | Spores/g. after gas | Spores/g. before gas | Spores/g. after gas |
| 4·5 | $1·1 \times 10^8$ | 2 | 450 | 70 |
| 1·3 | $8·6 \times 10^5$ | 3 | 500 | 63 |
| 0·27 | $9·2 \times 10^5$ | 0 | 680 | 93 |

The almost complete kill with the externally and heavily contaminated crystals, in contrast to the limited reduction of viable spores in those crystals isolated from contaminated solution, supported the conclusion drawn from the preceding experiment. Most of the contamination associated with crystals from a contaminated liquor was on the surface, and the amount of contamination, both internal and external, did not vary greatly with crystal size, provided that the conditions of growth were the same in each case. The resistant spores may be protected from the gas by lodgment in crevices or cracks in the crystal surface. Increasing the exposure time up to 80 hours did not improve the kill under the experimental conditions, as shown in Table III.

TABLE III

EFFECT OF PROLONGED EXPOSURE TO FORMALDEHYDE

| Time of exposure (hours) | Spores/g. of crystals | |
|---|---|---|
| | Contaminated externally | Grown in contaminated liquor |
| 0 | 10,000 approx. | — |
| 5 | 0 | 3780 |
| 20 | — | 4210 |
| 40 | — | 3830 |
| 60 | — | 4470 |
| 80 | — | 4160 |

*Influence of Rate of Crystallisation on Inclusion of Spores*

The more rapidly a crystal is grown the more likely it is to contain inclusions of impurities. If this applies equally to inclusions of viable spores the chances of such inclusion taking place in a precipitation process typical of manufacturing practice are increased. The following experiments show the differences found by crystallisation from the same liquor by (*a*) normal growth as described already, and (*b*) a precipitation process. Aliquots of the cooled sterile and contaminated liquors prepared as previously described were poured into a slightly larger volume of 70 per cent. ethanol, previously sterilised by filtration. This mixture was stirred until crystallisation occurred and the crystals isolated by filtration using a No. 2 sintered glass funnel and vacuum under an aseptic screen. The crystals were then dried in closed sterile dishes at 37° C. When dry they were sieved through a 30 mesh sieve. This gave free flowing powders which did not cake further during the subsequent gas procedure. The uncontaminated material was tested for sterility and if found to be sterile was contaminated by mixing with spore powder. From another aliquot of the contaminated liquors, crystals were also grown by the normal slow growing process. These procedures provided, (i) rapidly grown (precipitated) small crystals from sterile mother liquors, (ii) crystals from (i) above externally contaminated with freeze dried spore powder, (iii) rapidly grown (precipitated) small crystals from contaminated mother liquors, and (iv), slowly grown larger crystals from the same contaminated mother liquor as in (iii).

A total viable bacterial count was then made on these crystals using groups of ten weighed large crystals and groups of 10 × 100 mg. samples

of crystalline powder, before and after exposure to gas, and the results are given in Table IV.

Obviously the method of exposing the crystalline powders to gas was sterilising the outside of the powder, and the small crystals grown rapidly were more heavily contaminated than the crystals grown slowly. Rapid crystal growth from bacterially contaminated liquors, therefore, increased the risk of internal contamination of the crystals.

TABLE IV

RATES OF CRYSTALLISATION
SPORES/G. IN CRYSTALS PREPARED BY VARIOUS METHODS

| RAPID CRYSTALLISATION | | | | SLOW CRYSTALLISATION | |
|---|---|---|---|---|---|
| Externally contaminated | | From contaminated liquor | | From contaminated liquor | |
| Before gas | After gas | Before gas | After gas | Before gas | After gas |
| $1 \times 10^8$ approx. | 0 | $1{\cdot}3 \times 10^6$ | 5840 | 855 | 96 |

TABLE V

AMOUNT OF CONTAMINATION

| Initial count in liquor spores/g. | Powder after drying | |
|---|---|---|
| | Spores/g. before gas | Spores/g. after gas |
| 10,000 | 8130 | 620 |
| 500 | 605 | 99 |
| 50 | 80 | 17 |
| 10 | 10 | 1·2 |

*Amounts of contamination and spore survival.* The amount of contamination met in manufacturing practice will be likely to be lower than those used in the previous experiments. It was, therefore, of interest to ascertain the degree of contamination in the final product, knowing the actual degree of contamination in the mother liquors. Table V shows the contamination in crystalline Rochelle salt powders before and after exposure to gas, after various initial counts in the mother liquors.

At 10 organisms/g. of mother liquor the final bacterial count on the gassed powder was only 1·2 organisms/g. This low count necessitated the plating technique being applied on a larger scale, and a full 10 g. of powder (in 100 mg. samples) was tested.

*Survival of spores on storage.* Although the evidence so far presented shows that *B. subtilis* spores can survive inside crystals, the possibility arises that they may not survive prolonged storage. The number of bacteria surviving in samples of Rochelle salt crystals and crystalline powder prepared from contaminated mother liquor was, therefore, investigated after a period of storage at laboratory temperature (Table VI). In all tests the samples were exposed to gas before making the viable count. Both samples were still heavily contaminated at the end of the storage period.

*Other crystalline materials.* The evidence so far presented relates only to Rochelle salt. A further series of rapidly crystallised water-soluble

TABLE VI

SURVIVAL OF SPORES IN CRYSTALS ON STORAGE

| Material | Spores/g. | | Period of Storage in weeks |
|---|---|---|---|
| | Initially | After storage | |
| Crystals .. .. | 450 | 470 | 21 |
| Crystalline powder .. | 813 | 540 | 16 |

powders were prepared from both sterile and contaminated mother liquor and examined in a similar way, using formaldehyde. (See Table VII.)

These results confirm that in many compounds crystallised from contamined mother liquor, spores are included within the crystal and remain protected from the gaseous sterilisation procedure.

TABLE VII

SURVIVAL OF SPORES IN CRYSTALLINE MATERIALS

| Substance | Precipitant | Size and shape of crystals (dimensions in μ) | Viable spores/g. | | | | |
|---|---|---|---|---|---|---|---|
| | | | Grown from contaminated liquor | | | Contaminated externally | |
| | | | Mother liquor (calc.) | Before gas | After gas | Before gas (calc.) | After gas |
| Hexamine | Acetone | Aggregates, rectangular plates mixed | 1000 | 236 | 47 | $10^8$ | 0 |
| Ethylene diamine tartrate | Ethanol | Aggregates, needles and plates very mixed | 100 | 96 | 15 | $10^6$ | 0 |
| Lactose | Ethanol | Aggregates of needles and rhomboids, bulk 40–60 long | 1000 | 83 | 0·4 (2 in. 5 g.) | $10^6$ | 0 |
| Glycine | Ethanol | Aggregates, oblong 90 long × 30 wide × 30 | 19,000 | 6200 | 125 | $10^8$ | 0 |
| Sodium diphosphate | Acetone | Aggregates, needles 130–180 long, 6–15 wide | 10,000 | 10,000 | 1100 | $10^8$ | 0 |
| Hexamethonium bromide | Acetone | Aggregates rectangular tables 180–300 × 600 | 10,000 | 640 | 150 | $10^6$ | 0 |
| Sucrose | Evaporated from syrup | Aggregates of cubes, bulk 150–200 side | 10,000 | 0 | — | — | — |
| Chloral hydrate | Evaporated from ethereal solution | Aggregates no particular shape discernible, crystals in double pyramid | 5000 | 0 | — | — | — |
| Sodium chloride | Ethanol | Cubes, bulk about 250 side | 10,000 | 0 | — | — | — |

*Sterilisation with Ethylene Oxide*

To confirm the phenomenon of viable spore inclusion in crystalline materials, it was thought desirable to use an alternative gas to formaldehyde, and ethylene oxide was investigated. Because of its explosive nature the method of exposure was different. The samples were placed in test tubes connected to a common gas inlet tube. The entire apparatus

was evacuated on a water-pump for 10 minutes, flushed with carbon dioxide, again evacuated on the water pump for 15 minutes and then filled with ethylene oxide at atmospheric pressure. After standing for the required period of time, the apparatus was evacuated on the water pump for 5 minutes and flushed with carbon dioxide before removing the samples. In the test in which extra moisture was added, an extra test tube containing water was connected to the gas inlet tube. Four samples of Rochelle salt were exposed simultaneously, and the results are given in Table VIII.

TABLE VIII

EXPOSURE TO ETHYLENE OXIDE

| Material | Viable spores/g. of crystal | | | |
|---|---|---|---|---|
| | Initially | 24 hours exposure | 96 hours exposure | 96 hours exposure with extra moisture |
| Sterile crystals dusted with freeze dried spores | $1 \times 10^6$ approx. | — | 53 | 110 |
| Crystals grown from contaminated liquor | 6000 approx. | 5140 | 4870 | 2600 |
| Sterile powder dusted with freeze dried spores | $10 \times 10^6$ approx. | 172 | 24 | 4 |
| Powder crystallised from contaminated liquor | $1 \times 10^6$ approx. | 1960 | 6250 | 4490 |

While the maximum period of exposure to ethylene oxide was insufficient to sterilise surface-contaminated material, there was a reduction in the viable count. With both the large crystals and the crystalline powder prepared from contaminated mother liquor, the number of viable organisms/g. remained quite high, providing further evidence that viable spores are included in such materials.

## DISCUSSION

The results indicate that crystals contaminated on the external surface only can be sterilised by the described process using formaldehyde gas, but that crystals grown from contaminated liquors frequently include spores which are not sterilised by this process, even by extending the exposure time to 80 hours. It is probable that many are entrapped inside the crystal mass, and are, therefore, inaccessible to the gas. This theory is supported by the results of experiments in which ethylene oxide was the bactericidal gas and also by electron micrographs which show spore-like objects found on the fractured surfaces of contaminated crystals. (See Appendix.) It also appears that the more rapidly crystallisation takes place, the greater is the chance of inclusion of bacterial organisms. Furthermore, with *B. subtilis* spores, the organism is capable of surviving inside the crystal for at least five months. Most of this work was carried out with Rochelle salt, but experiments show that the inclusion phenomenon applies to many other materials. Exceptions were noted, in sucrose, chloral hydrate and sodium chloride crystals grown from contaminated liquors. The reason for this has not been investigated.

There is some evidence[1,2] that the possibility of the inclusion of small pockets of contaminated liquor in the crystals has to be considered. With these three substances it is known that the liquors possess strong osmotic properties, or even bactericidal powers on prolonged contact with spores. The inclusion effect in micro-crystals has not been investigated, although it seems obvious that, with small enough crystals, inclusion of a spore cannot take place. A 6-hour period was chosen for sterilising the exterior of contaminated crystals. In some subsequent experiments, occasional surface spores remained viable after this exposure period, but the numbers were not considered to affect the conclusions. An additional unexplained fact is that as shown in Table I; approximately 10 per cent. of the crystals grown from contaminated liquor did not contain viable organisms after exposure to formaldehyde gas, but subsequent quantitative tests did not result in a repetition of the observation. Comparatively heavy contamination was used in this work for convenience. Much less may be encountered in mother liquors during "clean" working as opposed to aseptic working, but the possibility still arises that crystals isolated from such liquors may be internally contaminated and, furthermore, the rapidity with which crystals are normally precipitated in manufacturing processes will increase this hazard. As a gas procedure cannot be relied upon to sterilise such crystals, the mother liquor must be sterile. Gas may, however, be useful in reducing external bacterial contamination, and thereby permitting a partial relaxation of aseptic precautions in the manufacturing stages after the sterile crystals have first been prepared. For these reasons, the control of gas procedures must be as rigid as control in normal aseptic processing, and it must not be assumed that exposure to gas can be compared with autoclaving or heat treatment. The design of the particular sterility test to be used is also of importance. Obviously if viable organisms inside crystals are to be detected the test must employ total solution of the solid, and sterility tests which involved the suspension of sparingly soluble solids in broth with subsequent subculturing should not be accepted as valid tests unless they indicate that the product is non-sterile. Whilst it has been shown that spores are protected from the sterilising effect of gases by inclusion in crystals, it is possible that other physical or chemical methods of sterilisation may be effective.

## APPENDIX

### *Examination of Crystals by Electron Microscopy*

Specimen crystals were prepared for examination by electron microscopy.

*Internally contaminated crystals* were crystallised from Rochelle salt solution prepared to contain about 5 million spores/g. *Control crystals* were prepared by aseptic crystallisation from liquor filtered through sintered glass 5 on 3 and autoclaved.

### B. subtilis *in Rochelle Salt*

*Specimen preparation for the electron microscope.* Since the crystals of Rochelle salt were too thick for direct examination in the transmission

type of electron microscope, they were viewed indirectly by means of a "replica" or "cast" of the surface formed in a suitable material and of sufficient thickness to be penetrated by the electron beam. A one-step negative replica technique was used in the form of an all-metal replica backed by a suitable polymer.

*Experimental details.* Heavily contaminated and uncontaminated, control crystals, 1 cm. in length and 0·5 cm. high, were selected, and scored around the outer surfaces at the centre with a sharp scalpel; this facilitated the splitting in half of the crystals. These pieces, with the

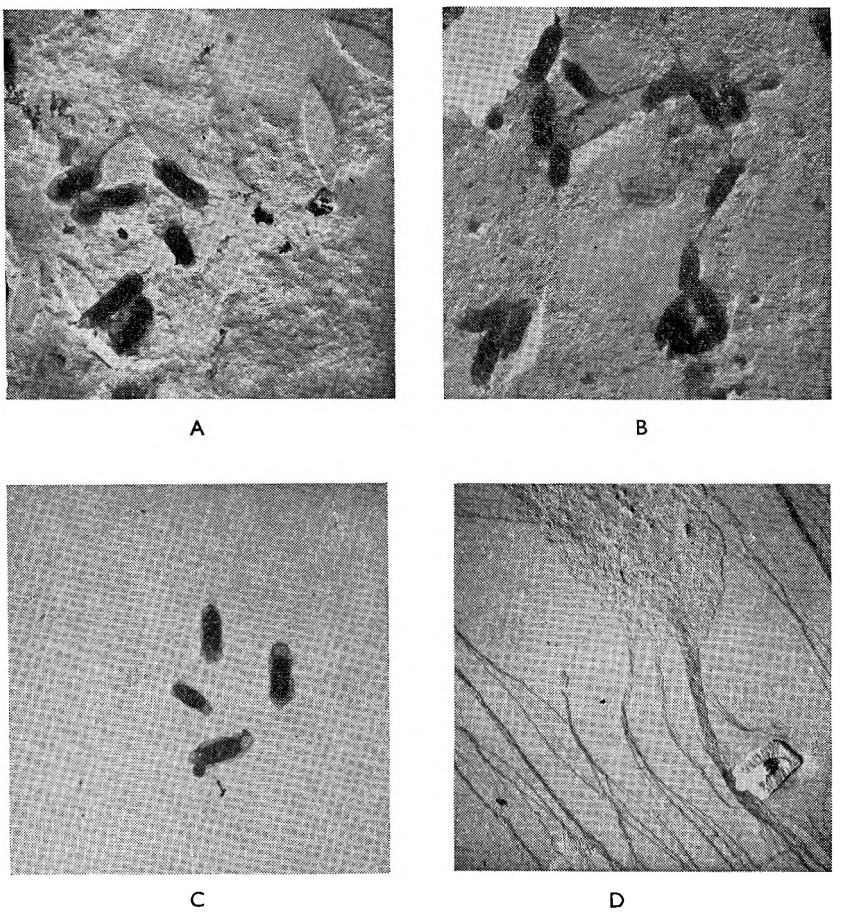


FIG. 2. Electron micrographs of crystal surfaces.

broken surfaces uppermost, were then placed on a microscope slide and chromium evaporated on to the surface *in vacuo* at a pressure of $10^{-5}$ mm. Hg. The angle of shadowing or evaporation was $\tan^{-1} 0{\cdot}2$. This surface was then coated with 0·25 per cent. nitrocellulose in amyl acetate, and when the solvent had completely evaporated the crystal was placed in a

petri dish. Water at 60° C. was carefully poured into the dish to the height of the crystal or until the film floated free, after which the crystal was removed immediately from the water before it completely dissolved. The replica was then washed twice in clean, distilled water to remove all traces of Rochelle salt, and mounted on 1/8 in. 250 mesh copper grids, examined, and electron micrographs taken in a Metrovick E.M. Type 2 at an electron magnification of × 2500.

*Observations.* On examination, pseudo-replicas had been produced since the majority of the bacteria had remained adhering to the metal-nitrocellulose film. Replicas from complementary broken surfaces showed aggregates of spores at fairly regular intervals throughout the specimen. To illustrate the imprisonment of bacteria within the crystal, areas where the spores were dispersed were selected and photographed. Specimens from the uncontaminated samples were thoroughly searched and no spores were observed.

Micrographs A, B and C are representative of broken crystal surfaces from Rochelle salt crystallised from liquor heavily contaminated with *B. subtilis* spores.

"D" is the main feature observed on many surfaces from sterile crystals.


## Summary

1. Crystals grown from contaminated mother liquors may remain contaminated after exposure to bactericidal gases. It seems that these spores are included within the crystal.
2. This phenomenon occurs with a sufficiently large number of compounds to indicate a general occurrence.
3. Speed of growth of the crystals has been found to influence the number of spores included.
4. Implications on manufacturing processes have been discussed.


The authors wish to acknowledge the co-operation and help of Dr. A. R. Martin with the ethylene oxide experiments, Mr. W. E. Durrant and Mr. R. T. Leah, Dyestuffs Division, I.C.I., for preparation and production of the electron micrographs, and Mr. A. G. Fishburn for assistance in the preparation of this paper.

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## DISCUSSION

The paper was presented by MR. J. COCKTON.

The CHAIRMAN asked whether the *B. subtilis* spores had been tested for resistance to formaldehyde, and whether there was any evidence that formaldehyde was taken up on the surface of crystals by absorption.

DR. W. MITCHELL (London) asked whether some of the organisms were sealed in the crystals and were therefore not accessible to the sterilising gas.

MR. J. A. MYERS (Bradford) enquired whether the authors would expect that calculi could contain embedded bacteria.

MR. J. COCKTON, in reply, said that the resistance of the *B. subtilis* spores to formaldehyde gas had not been measured. No evidence was found of adsorption of formaldehyde on the crystals. Removing the surface of the crystals in ethanol-formaldehyde, until half of the crystal weight had been dissolved showed viable spores still present. Electron micrographs seemed to suggest that the spores could be included within the matrix of the crystal. He saw no reason why spores should not be occluded in calculi.

# THE REPRODUCIBILITY OF EXTINCTION TIME ESTIMATES

## PART III. STUDIES OF SUSPENSIONS OF TEST ORGANISMS

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VARIATIONS in the resistance of suspensions or cultures of the test organism used in the evaluation of bactericides have been reported by several workers[1–3]. Cook and Steel[4] indicated that this variability might be reduced by the use of a single suspension, stored between experiments at room temperature.

The change in phenol resistance of suspensions during storage was investigated.

## EXPERIMENTAL

### *Preliminary Experiments*

The organisms used were, *Bacterium coli* (*Escherichia coli*), *Bacillus anthracis*, *Bordetella bronchisepticus*, *Mycobacterium smegmatis*, *Pseudomonas pyocyanea*, *Salmonella typhi*, *Shigella dysenteriae Type I*, *Staphylococcus aureus* and *Streptococcus fæcalis*. The strains used were those used by Cook[5].

### *Method*

The organisms were grown in Roux bottles on Lemco agar for 48 hours. Suspensions were made by washing the organisms off the agar with 50 ml. of sterile distilled water and were stored in 100 ml. bottles. No attempt was made to wash the suspensions free from agar or nutrients.

Counts were made of the suspensions using the Miles and Misra[6] technique with tenfold dilutions and five replicate plates for each organism.

Table I shows the results of the initial counts and the counts after storage for a month at ambient room temperature.

TABLE I

VIABLE COUNT OF SUSPENSIONS STORED AT ROOM TEMPERATURE FOR 1 MONTH

| Organism | Original count | Count after storage |
|---|---|---|
| *Bacterium coli* | $7{\cdot}9 \times 10^9$ | $5{\cdot}4 \times 10^8$ |
| *Bacillus anthracis* | $7{\cdot}4 \times 10^5$ | $6{\cdot}0 \times 10^4$ |
| *Bordetella bronchisepticus* | $2{\cdot}4 \times 10^{10}$ | $1{\cdot}0 \times 10^8$ |
| *Mycobacterium smegmatis* | $3{\cdot}0 \times 10^8$ | $1{\cdot}1 \times 10^7$ |
| *Pseudomonas pyocyanea* | $7{\cdot}6 \times 10^8$ | $5{\cdot}2 \times 10^7$ |
| *Salmonella typhi* | $8{\cdot}0 \times 10^9$ | $1{\cdot}8 \times 10^7$ |
| *Streptococcus fæcalis* | $3{\cdot}6 \times 10^8$ | $9{\cdot}0 \times 10^7$ |

Other experiments had indicated that a constant temperature of 10° C. might result in an increase of the numbers of organisms surviving. Fresh suspensions were made and stored at 10° ± 1° C. Counts were made at intervals on these suspensions.

The resistance of the organisms to several bacteriostatics were determined initially and after three months storage. This was done by inoculating agar plates containing various strengths of the bacteriostatics with drops of the suspensions, as described by Cook[5].

Figure 1 shows the reductions in the viable counts over the period of storage, and Table II shows changes in bacteriostatic resistance.

TABLE II

CHANGE IN BACTERIOSTATIC RESISTANCE OF VARIOUS ORGANISMS AFTER 3 MONTHS STORAGE

| | | *Bact. coli* | *B. anthracis* | *Bordetella bronchisepticus* | *Myco. smegmatis* | *Ps. pyocyanea* | *Salm. typhi* | *Shig. dysenteriæ* | *Staph. aureus* | *Strept. fæcalis* |
|---|---|---|---|---|---|---|---|---|---|---|
| Phenol | A | 0·2 | 0·2 | 0·1 | 0·1 | 0·2 | 0·2 | 0·2 | 0·1 | <0·1 |
| | B | 0·2 | 0·1 | 0·1 | 0·1 | 0·2 | 0·1 | 0·1 | 0·1 | <0·1 |
| Chloro-cresol | A | 0·04 | 0·04 | >0·02 | >0·02 | <0·08 | 0·04 | 0·04 | 0·04 | 0·08 |
| | B | 0·04 | >0·02 | >0·02 | >0·02 | 0·08 | 0·04 | >0·02 | 0·04 | 0·04 |
| Phenyl mercuric acetate | A | 0·002 | >0·0001 | 0·002 | >0·0001 | 0·002 | 0·002 | 0·002 | >0·0001 | 0·002 |
| | B | 0·0005 | >0·0001 | 0·0005 | >0·0001 | 0·002 | 0·0005 | >0·0001 | >0·0001 | >0·0001 |
| Cetrimide | A | >0·005 | >0·005 | >0·005 | >0·005 | >0·1 | 0·025 | >0·005 | >0·005 | 0·025 |
| | B | >0·005 | >0·005 | >0·005 | >0·005 | 0·1 | >0·005 | >0·005 | >0·005 | >0·005 |
| Crystal violet | A | 0·002 | 0·0005 | <0·002 | 0·002 | <0·002 | <0·002 | <0·002 | 0·0005 | 0·0005 |
| | B | 0·002 | >0·0001 | <0·002 | 0·0005 | <0·002 | <0·002 | <0·002 | 0·0001 | 0·0005 |
| Aminacrine hydrochloride | A | <0·08 | <0·08 | 0·016 | >0·002 | 0·08 | 0·016 | >0·002 | >0·002 | >0·002 |
| | B | <0·08 | <0·08 | >0·002 | >0·002 | 0·08 | >0·002 | >0·002 | >0·002 | >0·002 |
| Chloramine T | A | 0·5 | 0·2 | 0·5 | 0·5 | 0·5 | 0·5 | 0·2 | 0·5 | 0·5 |
| | B | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 |

A = inhibiting concentration (expressed as percentage) initially.
B = " " ( " " " ) after storage.

*Investigations of unwashed suspensions of* Bacterium coli *stored at different temperatures*

The strain of *Bacterium coli* used by Cook and Steel[4] had better survival figures on storage than any of the organisms used in the preliminary experiments and so it was decided to use this organism for further investigations.

The suspension was prepared by washing the 24 hour growth from several Roux bottles containing a peptone agar (1 per cent. peptone, 0·5 per cent. sodium chloride, 2 per cent. agar, pH 7·2). After shaking vigorously with glass beads, the suspension was centrifuged at 3000 r.p.m. for 1 minute, the supernatant removed and diluted to give an optical density equivalent to a total count of $2 \times 10^9$ per ml. Five ml. portions of the suspension were distributed in 5 ml. ampoules, which were sealed and divided into 5 batches for storage at temperatures of 4°, 10°, room temperature (c. 18° C.), 20° and 37° C. At intervals during the next six months, ampoules were opened and viable counts and extinction times determined by the methods outlined below.

The counting method used was that described by Miles and Misra[6]. A suitable number of drops of suspension was added to suitable volumes of sterile water. After thorough mixing, samples at two or more dilutions were dropped on to the surfaces of over-dried agar plates, the agar having the same composition as that used in preparation of the suspension. Dropping pipettes were of the same type and similar accuracy to those described by Cook and Yousef[7] and Cook[5]. Between 20 and 60 samples were taken at each dilution and the colonies were counted after incubation at 37° C. for between 12 and 15 hours.

Extinction times were determined by the method described by Berry and Bean[8] subject to the modifications put forward by Cook and Wills[3,9]. The bactericide solutions used contained 1·1 per cent. phenol in aqueous solution at 20° C. Each experiment consisted of between 10 and 20 replicate determinations—usually 15—and the results were expressed as mean single survivor times according to the analysis of Mather[10].

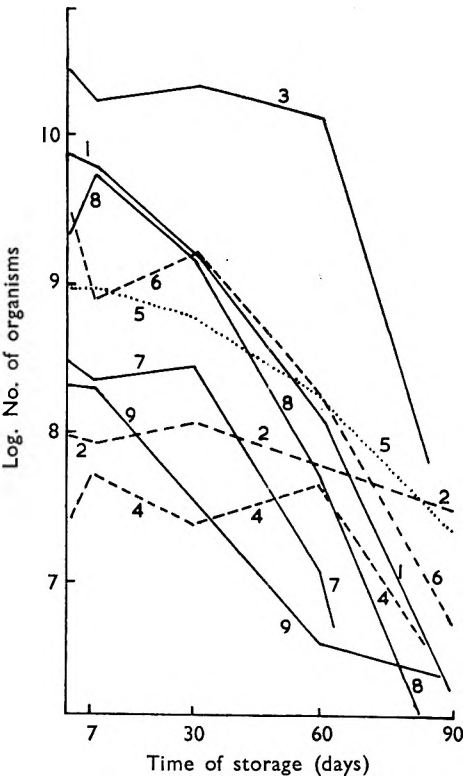


FIG. 1. Numbers of viable organisms per ml. in suspensions of different bacteria stored at 10° C. for various times. 1. *Bacterium coli*; 2. *Bacillus anthracis*; 3. *Bordetella bronchisepticus*; 4. *Mycobacterium smegmatis*; 5. *Pseudomonas pyocyanea*; 6. *Salmonella typhi*; 7. *Shigella dysenteriæ* (type I); 8. *Staphylococcus aureus*; 9. *Streptococcus fæcalis*.

The results of viable counts and extinction times of the suspension after varying periods of storage are shown in Table III. After the 23rd day of storage, the extinction times of the suspensions kept at 37° C. decreased so much that a lower phenol concentration of 0·7 per cent. had to be used in later experiments. The right hand column of the Table records extinction times to 1·1 per cent. phenol of a second laboratory strain of *Bact. coli* which was of lower phenol resistance and which was stored at room temperature in a stoppered bottle.

*Investigation of washed suspensions of* Bacterium coli *stored at different temperatures and in different containers*

The suspensions were prepared in the same way as before, but in

## TABLE III

VIABILITY AND EXTINCTION TIMES TO 1·1 PER CENT. AQUEOUS PHENOL SOLUTION OF UNWASHED SUSPENSIONS OF *Bact. coli* (N.C.T.C. 5933) STORED AT DIFFERENT TEMPERATURES

| Storage time (days) | 4° C. | | 10° C. | | Room temperature (*c.* 18° C.) | | 20° C. | | 37° C. | | Other strain (18° C.) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Viable count/ml. | Extinction time (min.) | Viable count/ml. | Extinction time (min.) | Viable count/ml. | Extinction time (min.) | Viable count/ml. | Extinction time (min.) | Viable count/ml. | Extinction time (min.) | |
| 0 | — | — | $1·94 \times 10^{9}$ | — | — | — | — | — | — | — | 13·2 |
| 1 | — | — | — | — | — | — | — | — | — | — | 16·2 |
| 2 | — | — | — | — | — | 32·8 | — | — | — | — | — |
| 3 | $1·77 \times 10^{9}$ | — | — | — | — | — | — | — | — | — | 15·7 |
| 6 | — | — | $1·89 \times 10^{9}$ | — | $1·85 \times 10^{9}$ | — | $1·81 \times 10^{9}$ | — | $1·44 \times 10^{8}$ | — | — |
| 7 | $1·86 \times 10^{9}$ | 28·2 | — | — | — | — | — | — | — | 19·6 | 10·3 |
| 9 | — | — | — | 23·4 | — | — | $1·95 \times 10^{9}$ | 43·4 | — | — | — |
| 13 | — | — | — | — | — | — | $1·68 \times 10^{9}$ | 31·4 | — | — | — |
| 17 | — | — | — | — | — | 31·5 | — | — | — | — | 9·05 |
| 20 | — | 21·0 | — | — | — | — | — | — | — | 2·67 | — |
| 23 | — | — | — | — | — | — | — | — | — | 1·55 | — |
| 28 | — | — | — | 28·3 | — | — | — | — | — | — | — |
| 37 | — | 33·4 | — | — | — | — | — | 32·0 | $1·04 \times 10^{7}$ | 34·8 | — |
| 42 | — | — | — | 30·4 | $1·62 \times 10^{8}$ | 21·2 | — | — | — | 19·9 | — |
| 57 | — | — | — | — | — | 23·6 | — | 14·7 | $7·08 \times 10^{5}$ | 10·8 | — |
| 69 | — | — | — | 26·5 | — | — | — | — | — | 6·63 | — |
| 72 | — | 20·2 | — | — | — | 26·0 | — | — | — | — | — |
| 78 | $9·20 \times 10^{8}$ | — | $1·53 \times 10^{8}$ | 22·1 | $5·90 \times 10^{7}$ | — | $4·62 \times 10^{8}$ | — | $2·76 \times 10^{5}$ | — | — |
| 90 | $9·20 \times 10^{8}$ | — | $1·73 \times 10^{8}$ | — | $3·99 \times 10^{7}$ | — | $1·74 \times 10^{7}$ | — | $2·88 \times 10^{5}$ | — | 11·0 |
| 115 | — | — | — | — | — | 17·4 | — | — | $1·88 \times 10^{6}$ | — | — |
| 118 | — | 17·6 | — | 21·4 | — | — | — | — | — | — | — |
| 125 | — | — | — | — | — | — | — | 8·00 | — | — | — |
| 136 | — | — | — | — | — | — | — | — | $1·05 \times 10^{5}$ | 4·90 | — |
| 139 | $5·55 \times 10^{8}$ | — | $9·35 \times 10^{7}$ | — | $1·25 \times 10^{7}$ | — | $6·79 \times 10^{8}$ | — | — | — | — |
| 155 | $8·34 \times 10^{8}$ | — | $8·96 \times 10^{7}$ | — | $1·03 \times 10^{7}$ | — | $6·04 \times 10^{8}$ | — | — | — | — |
| 170 | $2·70 \times 10^{8}$ | 13·6 | $6·74 \times 10^{7}$ | 16·4 | — | 9·90 | — | 4·85 | — | — | — |
| 175 | — | — | — | — | — | — | — | — | $1·35 \times 10^{5}$ | 5·63 | — |

addition the cells were finally washed by centrifuging and resuspending in water, repeating the process 4 times. The final suspension was stored at 4°, 10°, 20° and 37° C., in 100 ml. glass-stoppered bottles, approximately half filled, in 5 ml. ampoules containing 5 ml., and in 5 ml. ampoules which were filled with carbon dioxide after distribution of the suspension. In addition, a few ampoules were filled as nearly as possible to capacity with the suspension and a few were filled with only 1 ml. of suspension.

TABLE IV

VIABILITY AND EXTINCTION TIMES TO 1·1 PER CENT. AQUEOUS PHENOL SOLUTIONS OF WASHED SUSPENSIONS OF *Bact. coli* (N.C.T.C. 5933) STORED AT DIFFERENT TEMPERATURES

| | 4° C. | | 10° C. | | 20° C. | | 37° C. |
|---|---|---|---|---|---|---|---|
| Storage time (days) | Viable count/ml. | Extinction time (min.) | Viable count/ml. | Extinction time (min.) | Viable count/ml. | Extinction time (min.) | Viable count/ml. |
| 0 | — | — | $1·69 \times 10^{9}$ | — | — | — | — |
| 5 | — | — | $1·65 \times 10^{9}$ | — | $1·42 \times 10^{9}$ | — | $1·12 \times 10^{9}$ |
| 9 | — | — | — | — | — | — | $5·16 \times 10^{8}$ |
| 13 | — | — | $1·56 \times 10^{9}$ | — | $1·03 \times 10^{9}$ | — | — |
| 15 | — | — | — | — | — | — | $2·06 \times 10^{8}$ |
| 22 | — | — | $1·48 \times 10^{9}$ | — | $9·66 \times 10^{8}$ | — | $7·32 \times 10^{7}$ |
| 26 | — | — | — | 16·2 | — | — | — |
| 28 | — | — | — | — | — | 11·3 | — |
| 35 | — | — | $1·27 \times 10^{9}$ | — | $7·80 \times 10^{8}$ | — | $1·24 \times 10^{7}$ |
| 50 | $7·80 \times 10^{8}$ | 7·90 | — | — | — | — | — |
| 52 | — | — | — | 10·5 | — | — | — |
| 55 | — | — | — | — | — | 9·59 | — |
| 57 | — | — | $7·32 \times 10^{8}$ | — | $4·50 \times 10^{8}$ | — | $4·40 \times 10^{6}$ |

The values of extinction times and viable counts listed in Table IV refer only to suspensions stored in bottles. At the end of 2 months a comparison of counts at different temperatures and in different containers was made. The results are recorded in Table V.

*Investigation of washed suspensions of* Staphylococcus aureus *stored at different temperatures and in different containers*

The organism used in these tests was *Staphylococcus aureus* (Oxford) N.C.T.C. No. 6571. The suspension was prepared as described above, the organisms being harvested from a medium containing 1 per cent. Lab Lemco, 1 per cent. peptone, 0·5 per cent. sodium chloride and solidified with agar (pH 7·2). Samples of the suspension were stored at 10°, room temperature, 20° and 37° C., and in the same range of containers as described above. In addition, 250 ml. plugged conical flasks containing about 50 ml. of suspension were stored at 10° and 37° C., a mark being filed on each flask at the liquid level to allow frequent adjustment to replace water lost by evaporation. Media used both in extinction time determinations and in the viable counts contained 1 per cent. Lab Lemco in addition to peptone. The results in Table VII were obtained from suspensions stored in bottles. Results of viable counts in all types of container at 3 different temperatures after storage for 2 months are shown in Table VI.

The time taken for visible colonies to appear increased with increased duration of storage, so that an incubation period of at least 48 hours

## TABLE V

COMPARISON OF VIABLE COUNTS PER ML. OF WASHED *Bact. coli* SUSPENSIONS STORED UNDER DIFFERENT CONDITIONS FOR 57 DAYS

| Storage temperature | 10° C. | | | 20° C. | | | 37° C. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Container .. .. | Bottle | Ampoule | Ampoule under $CO_2$ | Bottle | Ampoule | Ampoule under $CO_2$ | Bottle | Full ampoule | 3/4 full ampoule | 1/5 full ampoule | Ampoule under $CO_2$ |
| Initial count .. | $7{\cdot}32 \times 10^8$ | $7{\cdot}68 \times 10^8$ | $1{\cdot}47 \times 10^7$ | $4{\cdot}50 \times 10^8$ | $2{\cdot}88 \times 10^8$ | $5{\cdot}76 \times 10^6$ | $4{\cdot}40 \times 10^6$ | $6{\cdot}72 \times 10^5$ | $2{\cdot}83 \times 10^6$ | $3{\cdot}73 \times 10^6$ | $2{\cdot}10 \times 10^2$ |
| Count* after 48 hrs. | — | — | $1{\cdot}27 \times 10^7$ | — | — | $2{\cdot}58 \times 10^7$ | — | $1{\cdot}52 \times 10^7$ | $8{\cdot}40 \times 10^6$ | $6{\cdot}54 \times 10^6$ | $3{\cdot}78 \times 10^7$ |

* Suspensions aerated, transferred to test tube and incubated for 48 hours at the temperature at which the ampoules were originally stored.

## TABLE VI

COMPARISON OF VIABLE COUNTS OF WASHED *Staph. aureus* SUSPENSIONS, STORED UNDER DIFFERENT CONDITIONS FOR 56 DAYS

| Storage temperature | 10° C. | | | | 20° C. | | | 37° C. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Container .. .. | Bottle | Plugged flask | Ampoule | Ampoule under $CO_2$ | Bottle | Ampoule | Ampoule under $CO_2$ | Bottle | Plugged flask | Full ampoule | 3/4 full ampoule | 1/5 full ampoule | Ampoule under $CO_2$ |
| Initial count .. | $1{\cdot}32 \times 10^8$ | $8{\cdot}90 \times 10^6$ | $2{\cdot}64 \times 10^6$ | $1{\cdot}41 \times 10^4$ | $2{\cdot}08 \times 10^8$ | $1{\cdot}49 \times 10^6$ | $6{\cdot}96 \times 10^3$ | $1{\cdot}34 \times 10^8$ | $5{\cdot}70 \times 10^6$ | $1{\cdot}39 \times 10^6$ | $5{\cdot}82 \times 10^5$ | $5{\cdot}70 \times 10^4$ | 0 |
| Count* after 48 hrs. | — | — | — | $1{\cdot}79 \times 10^4$ | — | — | $3{\cdot}6 \times 10^2$ | — | — | $1{\cdot}73 \times 10^6$ | $1{\cdot}10 \times 10^6$ | $<6 \times 10^3$ | 0 |

* Suspensions aerated, transferred to test tube and incubated for 48 hours at 37° C.

at 37° C. was required before counting the plates towards the end of the investigation. The counts were also complicated by the appearance of variant colonies which were smaller and less pigmented than those originally growing. Organisms from these colonies showed no differences in either morphology or in coagulase activity from those of the original type of colony. This change was first noted with the suspension stored at 37° C., and later appeared in those stored at room temperature and at 20° C., but was not observed in that kept at 10° C. up to the end of the second month.

TABLE VII

VIABILITY AND EXTINCTION TIMES TO 1·0 PER CENT. AQUEOUS PHENOL SOLUTIONS OF WASHED SUSPENSIONS OF *Staphylococcus aureus* (N.C.T.C. 6571) STORED AT DIFFERENT TEMPERATURES

| | 10° C. | | Room temperature (*c.* 18° C.) | | 20° C. | | 37° C. |
|---|---|---|---|---|---|---|---|
| Storage time (days) | Viable count/ml. | Extinction time (min.) | Viable count/ml. | Extinction time (min.) | Viable count/ml. | Extinction time (min.) | Viable count/ml. |
| 0 | $2{\cdot}42 \times 10^{8}$ | — | — | — | — | — | — |
| 5 | $1{\cdot}78 \times 10^{8}$ | — | — | — | $9{\cdot}0 \times 10^{8}$ | — | — |
| 8 | $1{\cdot}10 \times 10^{8}$ | 10·8 | — | — | $4{\cdot}75 \times 10^{8}$ | — | — |
| 12 | $5{\cdot}40 \times 10^{8}$ | — | $1{\cdot}50 \times 10^{8}$ | — | — | 9·80 | — |
| 14 | — | — | — | — | $2{\cdot}04 \times 10^{7}$ | — | — |
| 15 | — | — | — | 6·78 | — | — | — |
| 16 | — | — | — | — | — | — | — |
| 20 | $1{\cdot}04 \times 10^{8}$ | — | — | — | $1{\cdot}04 \times 10^{7}$ | — | — |
| 29 | — | — | $5{\cdot}10 \times 10^{7}$ | — | — | — | — |
| 30 | $3{\cdot}66 \times 10^{7}$ | 5·95 | — | — | — | — | — |
| 34 | — | — | — | — | $4{\cdot}20 \times 10^{7}$ | 3·36 | $2{\cdot}66 \times 10^{6}$ |
| 48 | — | — | $3{\cdot}84 \times 10^{7}$ | 3·89 | — | — | — |
| 56 | $1{\cdot}32 \times 10^{6}$ | — | — | — | $2{\cdot}08 \times 10^{8}$ | — | $1{\cdot}34 \times 10^{8}$ |

*Effect of change of storage temperature*

Examination of Tables III and IV shows that both viability and extinction times of *Bact. coli* fall more rapidly on storage of suspensions at 37° C. than at the lower temperatures. After storage of the unwashed suspension for a little over one month, several ampoules were removed from the 37° C. incubator and kept at room temperature. After further varying periods, ampoules of changed and unchanged storage temperature were opened and extinction times on exposure to 0·7 per cent. phenol were measured. Two months later, a further sample of ampoules was similarly removed from the incubator and, in this case, both extinction times and viable counts were determined. The results given in Table VIII show that extinction times of suspensions of changed storage temperature do not fall far below the values determined at the time of removal. From the few counts which were performed, it appears that the viability of the changed temperature suspension does not decrease with storage to the same extent as with suspensions maintained at 37° C. throughout.

*Growth in aqueous suspensions of* Bacterium coli

Suspensions of unwashed cells, which had been stored at 37° C. and 10° C. for 2 months, were centrifuged, and the supernatant liquids separately filtered to remove bacteria. The filtered supernatant from

TABLE VIII

EFFECT OF CHANGE OF STORAGE TEMPERATURE ON VIABILITY AND EXTINCTION TIMES OF UNWASHED SUSPENSIONS OF *Bact. coli*

| Age of suspension stored at 37° C. | Duration of continued storage at 37° C. (days) | Extinction time (minutes) | Viable count/ml. | Duration of continued storage at room temperature (days) | Extinction time (mins.) | Viable count/ml. |
|---|---|---|---|---|---|---|
| 34 days | 1 | 34·8 | — | — | — | — |
| | 8 | 20·0 | — | 8 | 32·2 | — |
| | 22 | 10·8 | — | 22 | 39·3 | — |
| | 35 | 6·63 | — | 36 | 37·4 | — |
| 57 days | 1 | 10·8 | — | — | — | — |
| | 11 | 6·63 | $2{\cdot}55 \times 10^5$ | 13 | 8·26 | — |
| | 78 | 4·90 | $1{\cdot}05 \times 10^5$ | 81 | 8·47 | $2{\cdot}02 \times 10^5$ |
| | 117 | 5·63 | $1{\cdot}35 \times 10^6$ | 117 | 8·45 | $2{\cdot}09 \times 10^5$ |

the suspension formerly stored at 10° C. was then added to the packed cells of the suspension formerly stored at 37° C. Similarly, the filtered supernatant from the suspension previously stored at 37° C. was added to the packed cells of the suspension which had been stored at 10° C. The new suspensions were stored at the same temperature as that at which the cells they had originally contained had been stored. Viable counts and extinction times to phenol solutions were determined after a further 7 days. It was found that the treated suspensions stored at 10° C. had not undergone any appreciable change as compared with untreated suspensions. Treated suspensions stored at 37° C., however, had undergone a great increase in viability (from $2{\cdot}55 \times 10^5$ to $2{\cdot}04 \times 10^8$ per ml.) and in extinction time (from about 11 minutes on exposure to 0·7 per cent. phenol to 25·5 minutes with 1·05 per cent. phenol). Viable counts were also made at short intervals beginning immediately after transference of the supernatant; the results showed that the increase in viability was gradual. The plot of logarithms of bacterial numbers against time (Fig. 2, curve A) resembles a growth curve.

Ampoules of unwashed suspension which had been stored at 37° C., were opened, the liquid aerated by bubbling sterile air from a capillary pipette, and the suspension transferred to a sterile test tube. On re-incubation, the viable count increased to almost the same extent as described above and as shown in Figure 2, curve B. Removal of the supernatant from suspensions stored at 37° C. and replacement with distilled water led to nearly the same increase in viable count after re-incubation (Fig. 2, curve C).

The second row of values in Tables V and VI relate to aerated and re-incubated suspensions; they illustrate the effect of type of container, volume of suspension contained and presence of carbon dioxide on the increase in viability which ensues.

## DISCUSSION

The preliminary results for the maintenance of viability and sensitivity to bacteriostatics of suspensions of several different organisms (Tables I and II, Fig. 1) show that more than 1 per cent. viability of all suspensions

was maintained for at least 2 months. A spore-forming organism, *Bacillus anthracis*, and an acid-fast organism, *Mycobacterium smegmatis*, underwent increases in viability over part of the period. The preliminary results, although for a different strain of *Bacterium coli* than that used by Cook and Steel[4], indicate that aqueous suspensions of this organism are not so stable as had originally been supposed. However, resistance to bacteriostatics is probably maintained more constantly with *Bact. coli* than with the other organisms used in this investigation and it was for this reason that *Bact. coli* was selected for more detailed study. It will be noticed from Table II that variations in resistance appear to develop more readily to crystal violet than to most other bacteriostatic substances which were examined.

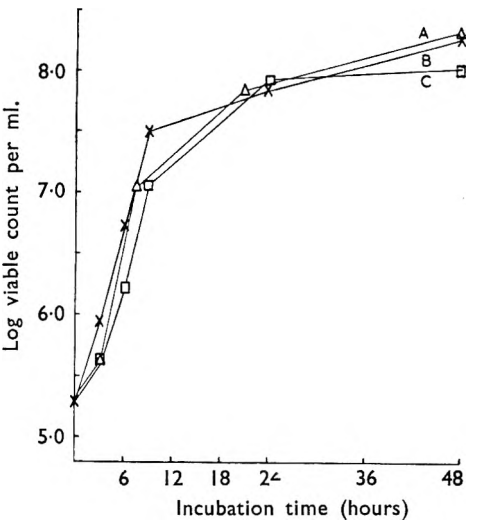


FIG. 2. Increase in viable count of suspensions of *Bacterium coli* stored at 37° C. for 2 months. Curve A (△——△) represents increases in viability when the cells of a suspension stored at 37° C. were resuspended in the supernatant of a suspension stored at 10° C. Curve B (×——×) was obtained on aeration and re-incubation of a suspension previously stored at 37° C. Curve C (□——□) represents the increase in viability when the cells of a suspension previously stored at 37° C. were resuspended in sterile distilled water.

Studies of the effects of temperature on the storage of unwashed suspensions of *Bact. coli* indicate that a greater stability is attained at temperatures at or below 10° C. The percentage survival and extinction times to 1·1 per cent. phenol solution (expressed as a per cent. of the original extinction time) after storage for 6 months were as follows.

| °C. | Viability per cent. | Extinction time per cent. |
|---|---|---|
| 4 | 13·9 | 41·5 |
| 10 | 3·47 | 50·0 |
| Room temperature | 0·53 | 30·2 |
| 20 | 0·31 | 14·8 |
| 37 | 0·007 | Not comparable |

The results were generally similar with the use of washed suspensions of *Bact. coli*, with the exception that a poorer survival is apparent at 4° C. Numbers of viable organisms per ml. of washed suspensions are shown plotted logarithmically against storage times at 3 separate storage temperatures in Figure 3. There is increase in slope with increase in temperature. The initial rate of fall in viability is not maintained for suspensions

stored at 37° C., and it is possible that, over a longer period of storage, the time-survival curves may be non-linear. The extinction times are lower than those obtained with unwashed suspensions of the same viability and it appears likely that thorough washing of the cells leads to a lower value of extinction time.

The values recorded in Table VII indicate that washed suspensions of *Staph. aureus* underwent a greater loss of viability and greater fall in extinction time than did those of *Bact. coli*. It would appear that temperature has a smaller influence on survival of staphylococci.

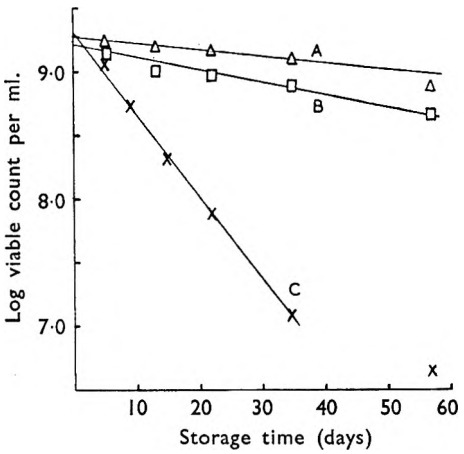


FIG. 3. Relationship between viability and storage time for the storage of washed suspensions of *Bacterium coli* at different temperatures. Curve A (△——△) stored at 10° C. Curve B (□——□) at 20° C. Curve C (×——×) at 37° C.

An indication of the most suitable containers for storage of suspensions of bacteria may be found from the values in Tables V and VI. Sealed ampoules seem to give lower survival than a bottle which is frequently opened to admit air. If it be assumed that the decay of suspensions at 37° C. constitutes only an acceleration of similar but much slower processes at lower temperatures, it may be concluded that suspensions may be stored in ampoules provided the volumes kept in the ampoules are small compared with the total capacity. The effects of type of container on the survival of *Staph. aureus* are much less certain.

The results throw some light on the possible mechanisms of survival of bacteria in water. It has been shown that *Bact. coli* surviving in the suspensions is able to increase in viability when suspended in its own ambient fluid, a filtrate of this fluid, or in distilled water. Viable counts after aeration and re-incubation (Table V) illustrate that the amount of regrowth is dependent upon the extent of decay of the suspension: the fewer the number of survivors, the greater the increase in viability which occurs afterwards. Where suspensions were stored at 37° C., decay was accelerated by storage under carbon dioxide, but the increase in viability after aeration and incubation was then the greatest. Stored in sealed ampoules under air, the rate of decay increased as the ampoule was more nearly filled and the increase in viability after aeration and re-incubation was found to increase correspondingly.

Two probable ways in which the increase in viability could be explained are (i) as a re-activation of cells presumed to be dead, or (ii) as actual growth of surviving bacteria. When the sterile supernatant from a

suspension was solidified with agar containing nutrients, there was no increase in the number of colonies from inocula of a stored suspension. This finding appears to contradict the hypothesis of re-activation. On the other hand, it was found that the increase in viability followed something very similar to a growth curve (Fig. 2). On the basis of all the evidence, it is considered that the process is probably one of growth, by which bacteria which die in the suspension are able to provide nutrient for use by the surviving organisms. That the process still took place when the cells were resuspended in water suggests that part at least of the nutrient is still associated with the dead cells and is not released into the suspension medium. Growth in such systems has been suggested by Steinhaus and Birkeland[11]. It will be noticed (Table VI) that a similar revival of *Staph. aureus* could not be demonstrated.

Survival of bacteria in stored suspensions can therefore be regarded as a dynamic process in which the survival of organisms is aided by those which have not survived. The survival rate is much decreased by either lack of oxygen or concentration of carbon dioxide and is dependent on temperature. Microscopical examination of stored suspensions did not show definite resting forms of *Bact. coli*. Cells were motile after several weeks but the cytoplasm appeared less dense than in freshly prepared suspensions.

It may be concluded that aqueous suspensions of bacteria may have application in testing bactericidal activity. *Bact. coli* appears to be a suitable organism for such application. If suspensions are stored at or below 20° C., they may be maintained for 1 month without appreciable decline in extinction times to aqueous phenol solutions; if stored at or below 10° C., suspensions may be kept for 2 or 3 months. If suspensions of constant viability are required, the period of safe storage is much shorter and probably does not exceed a few days at low temperature. The Oxford strain of *Staph. aureus* appears unsuitable for prolonged storage, but of the organisms employed in the preliminary investigation, *Salm. typhi* and other strains of *Staph. aureus* have a survival rate which is promising to the use of their stored suspensions in testing of disinfectants.

In a study of the factors which influence the reproducibility of extinction time estimates, Cook and Wills[3,12] concluded that variations among individual test suspensions was a major source of error. The use of stored suspensions may probably reduce this error, especially between different workers and laboratories. A method is provided by which extinction time determinations are made easier without sacrifice in accuracy.

## Summary

1. The survival of several different bacterial species in aqueous suspensions stored at 10° C. and at room temperature has been compared.

2. Changes in sensitivity to bacteriostatics of a range of organisms in stored suspensions has been studied. *Bact. coli* probably suffered less change in sensitivity than the other organisms tested.

3. Changes in viability and extinction times to phenol of washed and unwashed suspensions of *Bact. coli* and of washed suspensions of *Staph.*

*aureus* (Oxford strain) have been measured. The suspensions were stored at different temperatures and in various containers. Loss of viability and fall in extinction times were roughly parallel, were dependent on storage temperature and influenced by type of container.

4. Stored suspensions of *Bact. coli* at 4° and 10° C. will give consistent extinction times to phenol over periods of 2 or 3 months. Changes in viability occur after short periods of storage, and if stored suspensions are required to have a constant viability they may be kept for no more than a few days. Decrease in viability and extinction times of *Staph. aureus* suspensions was much more rapid.

5. Possible mechanisms of survival of bacteria in aqueous suspensions have been discussed.

The authors wish to thank Mr. A. Edwards and Miss R. McDonald for technical assistance.

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## DISCUSSION

The paper was presented by DR. B. A. WILLS.

The CHAIRMAN asked whether the authors considered that bacteria changed individually in their resistance; was there any evidence that some bacteria were dying and others replacing them? Had the authors carried out total counts to check whether there was an increase in the number of bacteria?

MR. J. A. VICKERS (Sunderland) pointed out that the organisms listed in Table I were counted by the Miles and Misra method, and asked whether any precautions were taken during the viable counting process to ensure that clumps of organisms were broken up.

DR. G. E. FOSTER (Dartford) said it seemed to him that there could not be more viable bacteria present than the number originally included.

MISS A. E. ROBINSON (London) said she noted that the survival of *E. coli* in stored suspensions was considered to be partly dependent on nutrients associated with the dead cells, and that this could not be shown in *Staph. aureus* suspensions. Was it possible that the chemical nature of the cell surface might account for this difference? Could the authors give further details of the standardisation of their bacterial suspensions since the optical density could be affected by a number of factors including the concentration of salts present.

DR. A. H. BECKETT (London) pointed out that as the authors had used only phenol as a bactericide it would be necessary to show that extinction times remained constant for each other bactericide examined before using the method, since bactericides exerted their effect by many different mechanisms.

DR. B. A. WILLS, in reply, said that a limited number of total counts of the bacteria in the suspensions was carried out, from which it appeared that the number of organisms remained approximately constant. The clumping of the organisms was not allowed for in the initial rough determinations which were made using suspensions of various organisms. The number of organisms did not increase in excess of the original number present. The number given was that originally present in the suspension as it was made. There might be an increase in the number present before reincubation was commenced but not beyond the number initially present. Due to the thorough washing of the suspensions they would remain unaffected by electrolyte concentration. He did not consider that the fact that the investigations had been restricted to one bactericide affected the validity of their conclusions. He agreed however that before one could start testing with other bactericides further work would be necessary using those bactericides.

# AN EXAMINATION OF RUBBER USED AS A CLOSURE FOR CONTAINERS OF INJECTABLE SOLUTIONS

## Part II. The Absorption of Chlorocresol

By W. T. Wing

*From the Pharmaceutical Department, Newcastle General Hospital*

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The examination of rubber has continued with the investigation of the absorption of chlorocresol. Many of the rubbers examined for phenol absorption[1] have been used and are referred to under the same sample numbers and their composition is given in Table I.

TABLE I

Particulars of rubber samples employed

| Sample number | Type of tubing | Rubber content, per cent. | Main filler | Other details |
|---|---|---|---|---|
| 1 | Red | 33 | Calcium carbonate | Laboratory tubing |
| 2 | Red | 50 | Calcium and magnesium carbonates | Drainage tubing |
| 3 | Latex | 90 | Magnesium carbonate | Transfusion tubing |
| 4 | Black | 50 | Carbon black | Anti-static tubing |
| 5 | Red | — | — | General purpose tubing |
| 6 | Red | — | — | " |
| 7 | Latex | — | — | Transfusion tubing |
| 8 | Latex-silicone | — | None | Vulcanising ingredients:—sulphur, zinc oxide and organic accelerator of dithio-carbamate series. Latex is pre-vulcanised and afterwards centrifuged to remove surplus vulcanising ingredients |
| 9 | Silicone | — | Silica | Transfusion tubing |
| 10 | Red | 50 | Calcium and magnesium carbonates | Drainage tubing |

## Experimental and Results

### *Estimation of Chlorocresol*

The method used, as with phenol, was based on the production of a blue colour with Folin-Ciocalteau reagent in alkaline solution, the intensity of which was estimated in a Spekker absorptiometer.

A calibration curve was produced using dilutions of an 0·2 per cent. solution of chlorocresol containing 0·032, 0·064, 0·096, 0·128, 0·16, 0·24 and 0·32 mg. per 4 ml. To each was added 0·6 ml. of Folin-Ciocalteau reagent and 1 ml. of 25 per cent. solution of sodium carbonate. The solutions were mixed, heated for 15 minutes in a water bath at 37° C. and readings obtained in a Spekker absorptiometer using filter number 608 with the test and blank solutions in 1 cm. cells.

When determining solutions which had been in contact with rubber, suitable dilutions were made to contain 0·05 to 0·12 mg. per 4 ml. when using the 1 cm. cell The concentration of chlorocresol was read from the calibration curve and the strength of the original solution obtained from the following equation.

$$\text{Chlorocresol concentration (percentage w/v)} = \frac{\text{No. of mg. per 4 ml. of dilution} \times 25 \times \text{dilution}}{1000 \times \text{thickness of cell in cm.}}$$

*Rate and Extent of the Absorption of Chlorocresol*

Rubber sample No. 10 was used to investigate the effect of time, temperature and the concentration of chlorocresol solutions upon rate and degree of absorption.

Tubes containing approximately 2 g. of rubber immersed in 10 ml. of 0·1 and 0·2 per cent. solutions of chlorocresol were flame sealed. Half were placed in a refrigerator at 2° C. and half in an incubator at 37° C., pairs being removed at intervals and analysed for chlorocresol content. Controls without rubber were similarly treated.

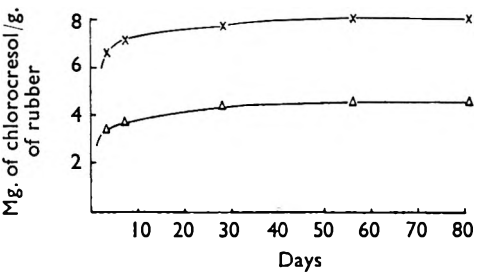


FIG. 1. Rate and amount of chlorocresol absorbed by rubber sample 10 when immersed in chlorocresol solutions of ×—× = 0·2 per cent. and △—△ = 0·1 per cent. at 2° C.

Table II shows that whereas the absorption of chlorocresol by rubber is rapid at 2° C. and about 75 per cent. of the final absorption is achieved in 3 days, complete absorption is not reached until about 7 weeks after contact with the solution. This is shown more clearly in Figure 1.

TABLE II

CHLOROCRESOL CONTENT OF RUBBER AND IMMERSING SOLUTIONS AFTER INTERVALS OF STORAGE AT 2° C.

| Chlorocresol content of original solution per cent. w/v | Storage time | Weight of rubber g. | Chlorocresol in rubber mg. | Chlorocresol in 10 ml. of solution mg. |
|---|---|---|---|---|
| 0·1 | 86 hours | 1·96 | 6·95 | 3·05 |
| | 181 hours | 1·99 | 7·39 | 2·61 |
| | | 2·01 | 7·35 | 2·64 |
| | 28 days | 1·97 | 8·69 | 1·24 |
| | | 1·96 | 8·69 | 1·24 |
| | 58 days | 2·02 | 9·22 | 0·88 |
| | | 1·99 | 9·22 | 0·88 |
| | 81 days | 2·01 | 9·22 | 0·88 |
| 0·2 | 86 hours | 1·99 | 13·2 | 6·8 |
| | 181 hours | 2·02 | 14·83 | 5·17 |
| | | 2·04 | 14·40 | 5·60 |
| | 28 days | 2·00 | 15·98 | 2·62 |
| | | 2·01 | 15·72 | 2·88 |
| | 58 days | 2·04 | 18·6 | 1·90 |
| | | 1·99 | 18·6 | 1·90 |
| | 81 days | 1·99 | 18·65 | 1·85 |

Table III shows that absorption is almost complete in one day when stored at 37° C.

The partition coefficients at equilibrium

$$K_{0\cdot1}^{2^\circ\,C.} = 62,\ K_{0\cdot2}^{2^\circ\,C.} = 59,\ K_{0\cdot1}^{37^\circ\,C.} = 42{\cdot}9,\ K_{0\cdot2}^{37^\circ\,C.} = 42{\cdot}9$$

TABLE III

CHLOROCRESOL CONTENT OF RUBBER AND IMMERSING SOLUTIONS AFTER INTERVALS OF STORAGE AT 37° C.

| Chlorocresol content of original solution per cent. w/v | Storage time | Weight of rubber g. | Chlorocresol in rubber mg. | Chlorocresol in 10 ml. of solution mg. |
|---|---|---|---|---|
| 0·1 | 1 day | 2·06 | 8·62 | 1·38 |
| | | 2·03 | 8·62 | 1·38 |
| | 3 days | 2·06 | 8·73 | 1·28 |
| | | 2·06 | 8·73 | 1·28 |
| | 7 days | 2·03 | 8·68 | 1·32 |
| | | 2·00 | 8·63 | 1·37 |
| | 23 days | 2·01 | 8·61 | 1·20 |
| | | 2·00 | 8·62 | 1·19 |
| 0·2 | 1 day | 2·04 | 17·15 | 2·85 |
| | | 1·99 | 17·05 | 2·95 |
| | 3 days | 1·97 | 17·33 | 2·67 |
| | 7 days | 2·00 | 17·28 | 2·72 |
| | | 2·00 | 17·33 | 2·67 |
| | 23 days | 2·03 | 17·24 | 2·38 |
| | | 1·99 | 17·28 | 2·34 |

show that whereas the amount of chlorocresol absorbed was directly proportional to the concentration of the immersing solutions, this was less at 37° C. than at 2° C. being approximately 86 per cent. and 92 per cent. respectively.

TABLE IV

PARTITION COEFFICIENTS FOR RUBBER SAMPLES 1 TO 9

| Sample | Specific gravity or rubber | K 2° C. 0·1 | | K 2° C. 0·2 | | K 37° C. 0·1 | | K 37° C. 0·2 | |
|---|---|---|---|---|---|---|---|---|---|
| | | Readings | Mean | Readings | Mean | Readings | Mean | Readings | Mean |
| 1 | 1·37 | 37·2<br>36·3 | 36·7 | 36·8<br>36·8 | 36·8 | 30·2<br>33·2 | 31·7 | 33·1<br>33·0 | 33·0 |
| 2 | 1·22 | 52·8<br>53·6 | 53·2 | 55·9<br>56·1 | 56·0 | 39·5<br>40·9 | 40·2 | 46·3<br>47·5 | 46·0 |
| 3 | 0·994 | 26·7<br>27·2 | 26·8 | 25·0<br>24·4 | 24·7 | 24·8<br>26·2 | 25·5 | 22·8<br>22·7 | 22·7 |
| 4 | 1·176 | 39·1<br>40·2 | 39·7 | 33·0<br>34·1 | 33·5 | 27·7<br>27·3 | 27·5 | 24·4<br>21·7 | 23·0 |
| 5 | 1·112 | 35·9<br>35·6 | 35·7 | 33·4<br>33·8 | 33·6 | 25·4<br>26·0 | 25·7 | 24·8<br>24·9 | 24·8 |
| 6 | 1·125 | 40·2<br>39·7 | 39·9 | 40·2<br>39·7 | 39·9 | 29·6<br>29·9 | 29·8 | 29·5<br>29·0 | 29·3 |
| 7 | 0·923 | 16·6<br>16·7 | 16·6 | 14·4<br>14·8 | 14·6 | 14·7<br>14·9 | 14·8 | 12·2<br>12·5 | 12·3 |
| 8 | 0·915 | 14·1 | 14·1 | 18·2<br>18·5 | 18·3 | 14·5<br>14·8 | 14·6 | 14·2<br>14·5 | 14·3 |
| 9 | 1·176 | 7·1<br>7·2 | 7·1 | 7·1<br>6·9 | 7·0 | 6·3<br>6·6 | 6·4 | 6·5<br>6·5 | 6·5 |

*The Partition Coefficients of Chlorocresol for Several Rubbers*

The same series of rubbers 1 to 9 mentioned in the previous paper[1] were examined to determine the amount of chlorocresol absorbed under several set conditions. Tubes containing approximately 2 g. of rubber were immersed in 10 ml. of 0·1 per cent. or 0·2 per cent. chlorocresol solution, heat sealed, and stored either at 2° C. or 37° C. The solutions

were examined after 55 and 21 days respectively. In Table IV are given the partition coefficients for chlorocresol where

$$K_c^t = \frac{Cr}{Cw} \text{ where } K = \text{partition coefficient}$$

at temperature $t$ determined for rubber immersed in chlorocresol solution of strength $c$ expressed as percentage w/v; $Cr$ = concentration of chlorocresol in rubber expressed as mg./ml.; $Cw$ = concentration of chlorocresol in water expressed as mg./ml.

It is seen that in all instances there is a higher absorption of chlorocresol at 2° C. than at 37° C., but that variation of the concentration of the immersing solutions, between 0·1 and 0·2 per cent. has little effect upon the partition coefficient. The proportion of chlorocresol absorbed is much higher than with phenol. For silicone tubing it is just over 50 per cent., for translucent latex rubber tubings between 73 and 80 per cent. and for other rubbers up to 91 per cent. This results in partition coefficients which are approximately 20 times greater than for phenol with the same rubber.

Berry[2] has also mentioned a similar degree of absorption from 0·1 per cent. solutions of chlorocresol.

## Summary

1. The absorption of chlorocresol from aqueous solution by rubber has been shown to proceed to a state of equilibrium under controlled conditions of storage.

2. The amount of chlorocresol absorbed bears a direct relation to the concentration of chlorocresol in the solution in contact with the rubber.

3. The rate of absorption increases with rise of temperature.

4. The amount absorbed is less at 37° C. than at 2° C.

5. The amount absorbed varies with the type of rubber.

## References

1. Wing, *J. Pharm. Pharmacol.*, 1955, **7**, 649.
2. Berry, *ibid.*, 1953, **5**, 1014.

# AN EXAMINATION OF RUBBER USED AS A CLOSURE FOR CONTAINERS OF INJECTABLE SOLUTIONS

## PART III. THE EFFECT OF THE CHEMICAL COMPOSITION OF THE RUBBER MIX ON PHENOL AND CHLOROCRESOL ABSORPTION

BY W. T. WING

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OWING to the variable composition of rubber it is possible that certain constituents in the mix may increase or decrease the absorption of phenol or chlorocresol. To determine whether this is so, several rubbers of known composition have been examined in which the fillers, accelerators and vulcanisers have been varied.

### EXPERIMENTAL AND RESULTS

*Effect of Fillers*

Eight rubber mixes were prepared containing three fillers having the possibility of chemical interaction, namely zinc oxide, magnesium carbonate and calcium carbonate and four which exert a reinforcing action to the rubber matrix, namely china clay, VN3 (a precipitated silica), lampblack and philblack, the remaining mix containing the curing ingredients only. All the mixes were vulcanised in a press for 12 minutes at 60 lb. per square inch steam pressure (approx. 153° C.). The compositions are given in Table I.

TABLE I

COMPOSITION OF RUBBER SAMPLES 11–18

| Sample number | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|
| *Ingredient by weight*— | | | | | | | | |
| Smoked sheet rubber .. | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Sulphur .. .. .. | 2·5 | 2·5 | 2·5 | 2·5 | 2·5 | 2·5 | 2·5 | 2·5 |
| Santocure .. .. | 1·25 | 1·25 | 1·25 | 1·25 | 1·25 | 1·25 | 1·25 | 1·25 |
| Zinc oxide .. .. | 5 | 25 | 5 | 5 | 5 | 5 | 5 | 5 |
| VN3-Silica .. .. | — | — | 38 | — | — | — | — | — |
| Calcium carbonate .. | — | — | — | 52 | — | — | — | — |
| Magnesium carbonate .. | — | — | — | — | 44 | — | — | — |
| Lampblack .. .. | — | — | — | — | — | 36 | — | — |
| Philblack .. .. | — | — | — | — | — | — | 36 | — |
| China clay .. .. | — | — | — | — | — | — | — | 52 |

Santocure is *N-cyclo*hexyl-2-benzothiazol sulphenamide which splits at vulcanisation temperature into mercaptobenzothiazole and *cyclo*hexylamine.

Approximately 2 g. of each sample of rubber was placed in tubes containing 10 ml. of 0·5 per cent. phenol solution or 0·1 per cent. chlorocresol solution and the tubes sealed. The rubber in phenol solutions was stored for 14 days at 37° C. and that in chlorocresol solutions stored at 37° C. for 23 days in order to establish equilibrium between the rubber mix and the solution. All were then opened and determinations made of the phenol or chlorocresol content of the solutions. In all instances the partition coefficients were calculated for each sample (Table II).

TABLE II

PARTITION COEFFICIENTS FOR RUBBER SAMPLES 11–18

| Sample number | Specific gravity of rubber | $K_{0.5}^{37^\circ C.}$ for phenol | | $\Sigma_{0.1}^{37^\circ C.}$ for chlorocresol | |
|---|---|---|---|---|---|
| | | Readings | Mean | Readings | Mean |
| 11 | 0·935 | 0·87<br>0·84 | 0·86 | 15·5<br>14·9 | 15·2 |
| 12 | 1·068 | 0·89<br>0·82 | 0·86 | 14·3<br>14·7 | 14·5 |
| 13 | 1·055 | 1·21<br>1·17 | 1·19 | 22·8<br>24·1 | 23·4 |
| 14 | 1·172 | 0·91<br>0·93 | 0·92 | 17·3<br>17·3 | 17·3 |
| 15 | 1·118 | 0·84<br>0·83 | 0·84 | 12·6<br>12·6 | 12·6 |
| 16 | 1·058 | 0·78<br>0·80 | 0·79 | 16·6<br>17·3 | 17·0 |
| 17 | 1·027 | 0·85<br>0·87 | 0·86 | 17·8<br>17·5 | 17·6 |
| 18 | 1·162 | 0·98<br>0·78 | 0·89 | 15·0<br>14·9 | 15·0 |

It is seen that apart from the VN3—a precipitated silica—which results in a higher degree of absorption, the addition of the fillers mentioned do not influence the amount of absorption to any marked extent.

A further series of rubber mixes were prepared to show whether variation in the amount of filler had any effect on the phenol or chlorocresol absorption. One sample contained no filler, the others variable amounts of VN3, and calcium carbonate as shown in Table III.

TABLE III

COMPOSITION OF RUBBER SAMPLES 19–24 PREPARED BY VULCANISING AT 60 LB. PER SQUARE INCH STEAM PRESSURE (APPROX. 153° C.) FOR 12 MINUTES

| Sample number | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|
| Pale crepe rubber .. | 100 | 100 | 100 | 100 | 100 | 100 |
| Zinc oxide .. .. | 5·0 | 5·0 | 5·0 | 5·0 | 5·0 | 5·0 |
| Sulphur .. .. .. | 2·5 | 2·5 | 2·5 | 2·5 | 2·5 | 2·5 |
| Santocure .. .. | 1·25 | 1·25 | 1·25 | 1·25 | 1·25 | 1·25 |
| VN3 .. .. .. | — | 10·0 | 40·0 | — | — | — |
| Calcium carbonate .. | — | — | — | 10·0 | 20·0 | 40·0 |

The rubber samples 19–24 were subjected to storage under the same conditions as samples 11–18, and the partition coefficients for each were calculated. These are shown in Table IV.

The results show that VN3 used as a filler increases phenol and chlorocresol absorption and is related to the amount of filler present. On the other hand calcium carbonate has little or no effect on this property even when the calcium carbonate content of the mix varies considerably.

The composition of rubber samples 25–34 is given in Table V.

*Effect of the Variation in Sulphur Content*

Four samples 25, 26, 27 and 19 differing only in the sulphur content were stored with phenol and chlorocresol solutions under the same

conditions mentioned above. The partition coefficients which are given in Table VI were calculated.

This shows that although the degree of absorption of chlorocresol rose slightly with an increase of the sulphur content of the mix, variation

TABLE IV

PARTITION COEFFICIENTS FOR RUBBER SAMPLES 19–24

| Sample number | Specific gravity of rubber | $K_{0.5}^{37°\,C.}$ for phenol | | $K_{0.1}^{37°\,C.}$ for chlorocresol | |
|---|---|---|---|---|---|
| | | Readings | Mean | Readings | Mean |
| 19 | 0·967 | 0·74<br>0·78 | 0·76 | 11·5<br>11·7 | 11·6 |
| 20 | 1·002 | 0·90<br>0·90 | 0·90 | 16·9<br>17·4 | 17·2 |
| 21 | 1·13 | 1·21<br>1·27 | 1·24 | 28·5<br>29·2 | 28·9 |
| 22 | 1·02 | 0·72<br>0·74 | 0·73 | 12·4<br>12·4 | 12·4 |
| 23 | 1·07 | 0·75<br>0·77 | 0·76 | 10·9<br>11·7 | 11·3 |
| 24 | 1·152 | 0·73<br>0·77 | 0·75 | 10·9<br>11·2 | 11·1 |

TABLE V

COMPOSITION OF RUBBER SAMPLES 25–34

| Sample number | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 |
|---|---|---|---|---|---|---|---|---|---|---|
| *Ingredients by weight* | | | | | | | | | | |
| Pale crepe rubber .. | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Zinc oxide .. .. | 5·0 | 5·0 | 5·0 | 5·0 | 5·0 | 5·0 | 0·5 | 0·75 | 1·0 | 1·5 |
| Santocure .. .. | 1·25 | 1·25 | 1·25 | — | — | — | — | — | — | — |
| Sulphur .. .. | 1·0 | 1·5 | 2·0 | 1·5 | 1·5 | 1·5 | 1·5 | 1·5 | 1·5 | 1·5 |
| P.T.D. .. .. | — | — | — | — | 0·4 | — | 0·4 | 0·4 | 0·4 | 0·4 |
| M.S. .. .. | — | — | — | 0·4 | — | — | — | — | — | — |
| M.B.T.S. .. .. | — | — | — | — | — | 0·125 | — | — | — | — |
| T.M.T. .. .. | — | — | — | — | — | 0·25 | — | — | — | — |

P.T.D. = Dipentamethylenethiuram disulphide.
M.S. = Tetramethylthiuram monosulphide.
M.B.T.S. = Benzothiazyl disulphide which breaks down to mercaptobenzothiazole.
T.M.T. = Tetramethylthiuram disulphide.

Samples 28, 29 and 30 were cured for 6 minutes at 60 lb. per square inch steam pressure. Samples 25–27 and 31–34 were cured for 12 minutes at the same steam pressure.

TABLE VI

PARTITION COEFFICIENTS FOR RUBBER SAMPLES 19, 25, 26 AND 27

| Sample number | Parts of sulphur | Specific gravity of rubber | $K_{0.5}^{37°\,C.}$ for phenol | | $K_{0.1}^{37°\,C.}$ for chlorocresol | |
|---|---|---|---|---|---|---|
| | | | Readings | Mean | Readings | Mean |
| 25 | 1·0 | 0·963 | 0·75 | 0·75 | 9·11<br>9·41 | 9·26 |
| 26 | 1·5 | 0·934 | 0·73<br>0·73 | 0·73 | 9·25<br>9·27 | 9·26 |
| 27 | 2·0 | 0·976 | 0·74<br>0·77 | 0·76 | 11·2<br>11·5 | 11·4 |
| 19 | 2·5 | 0·967 | 0·74<br>0·78 | 0·76 | 11·5<br>11·7 | 11·6 |

of the latter from 1 to 2·5 parts of sulphur had little influence upon the extent of phenol and chlorocresol absorption.

*Effect of the Variation of Zinc Oxide Content*

Samples 31, 32, 33, 34 and 29 containing between 0·5 and 5·0 parts of zinc oxide were stored with phenol and chlorocresol solutions under the same conditions mentioned above. The partition coefficients are given in Table VII.

TABLE VII

PARTITION COEFFICIENTS FOR RUBBER SAMPLES 31, 32, 33, 34 AND 29

| Sample number | Parts of zinc oxide | Specific gravity of rubber | $K^{37^\circ C.}_{0\cdot5}$ for phenol | | $K^{37^\circ C.}_{0\cdot1}$ for chlorocresol | |
|---|---|---|---|---|---|---|
| | | | Readings | Mean | Readings | Mean |
| 31 | 0·5 | 0·933 | 0·94<br>0·88 | 0·91 | 18·5<br>18·7 | 18·6 |
| 32 | 0·75 | 0·931 | 0·88<br>0·91 | 0·89 | 18·2<br>18·4 | 18·3 |
| 33 | 1·0 | 0·936 | 0·88<br>0·87 | 0·88 | 18·2<br>17·8 | 18·1 |
| 34 | 1·5 | 0·938 | 0·85<br>0·87 | 0·86 | 18·2<br>17·8 | 18·0 |
| 29 | 5·0 | 0·966 | 0·82<br>0·87 | 0·85 | 17·6<br>17·4 | 17·5 |

The results show a very slight decrease in phenol and chlorocresol absorption with an increase in the zinc oxide content and such that a variation in the zinc oxide content can only be regarded as having no significant effect. A similar observation can be seen with samples 11 and 12 in which 5 and 25 parts of zinc oxide are present by weight and the accelerator used was Santocure.

TABLE VIII

PARTITION COEFFICIENTS FOR SAMPLES 26, 28, 29 AND 30

| Sample number | Accelerator | Specific gravity of rubber | $K^{37^\circ C.}_{0\cdot5}$ for phenol | | $K^{37^\circ C.}_{0\cdot1}$ for chlorocresol | |
|---|---|---|---|---|---|---|
| | | | Readings | Mean | Readings | Mean |
| 26 | Santocure | 0·934 | 0·73<br>0·73 | 0·73 | 9·25<br>9·27 | 9·27 |
| 28 | M.S.* | 0·968 | 0·81<br>0·86 | 0·84 | 16·2<br>16·3 | 16·3 |
| 29 | P.T.D.* | 0·966 | 0·82<br>0·87 | 0·85 | 17·6<br>17·4 | 17·5 |
| 30 | M.B.T.S.*<br>T.M.T. | 0·968 | 0·80<br>0·78 | 0·79 | 16·2<br>16·1 | 16·2 |

* See footnote to Table V for nomenclature.

*Effect of the Use of Various Accelerators*

Three rubber mixes were prepared containing as accelerators (1) di-pentamethylenethiuram disulphide, (2) tetramethylthiuram monosulphide and (3) a mixture of benzothiazyl disulphide, which breaks down to mercaptobenzothiazole, and tetramethylthiuram disulphide. They were

cured for 6 minutes at 60 lb. per square inch steam pressure (approx. 153° C.).

The samples 28, 29 and 30, whose composition is given in Table V, were subject to storage in contact with phenol and chlorocresol solutions and the partition coefficients calculated. These are given in Table VIII and are compared also with sample 26 in which Santocure was used as accelerator but which was subject to 12 minutes curing at 60 lb. steam pressure.

There is little difference between the three mixes 28, 29 and 30 in phenol and chlorocresol absorption but they all show a higher absorption than the mix in which Santocure was used as the accelerator, although the curing time was twice as long for the latter.

*Effect of a Plasticiser*

A rubber mix of the same composition as sample 11 to which had been added 20 parts by weight of factice (an oxidation product of linseed oil), gave partition coefficients $K_{0\cdot5}^{37°\,C.}$ for phenol of 1·66 and $K_{0\cdot5}^{37°\,C.}$ for chlorocresol of 28·4. These results, confirmed by others where factice was a constituent, have shown that the presence of this substance causes a marked increase in phenol and chlorocresol absorption.

## Discussion

The absorption of phenol and chlorocresol by rubber is unaffected to a large degree by the variation of the chemical composition of the mix. By comparing samples 11 and 12 where the relative sulphur and zinc oxide content varies considerably and yet the absorption remains practically constant, it can be seen that the difference between series 25–27 and 19, in which the sulphur content varies and series 31–34 and 29, in which the zinc oxide content varies, is obviously due to the accelerators Santocure and dipentamethylenethiuram disulphide which were used respectively rather than to varying sulphur and zinc oxide contents. Again, by reference to samples 25–34, in which 4 different accelerators were used and the curing time varied from 6 to 12 minutes, it is seen that although an increase in zinc oxide resulted in a slightly lower absorption, the more important difference was the lower absorption resulting from using Santocure when compared with the other acclerators rather than in the length of time of the cure.

The use of the plasticiser factice causes a marked increase in absorption by the rubber as also did the filler VN3. Of all other substances used none seemed to affect the absorption markedly, but it is important to note than by comparing samples 11 and 19 which differed only in the rubber employed in the mix, that absorption variation may also arise from the type of crude rubber used in the mix.

## Summary

1. Rubbers of simple composition have been examined to determine whether variation of composition of the main types of ingredient cause any variation in the absorption of phenol or chlorocresol.

2. Rubber containing the fillers zinc oxide, calcium carbonate, magnesium carbonate, lampblack, philblack and china clay shows little variation from rubber containing no filler in their phenol and chlorocresol absorption.

3. The presence of VN3—a precipitated silica, results in an increased absorption which is related to the VN3 content.

4. An increase in sulphur content results in a slightly higher absorption.

5. An increase in zinc oxide content results in a slightly decreased absorption.

6. The accelerator Santocure (*N-cyclo*hexyl-2-benzothiazol sulphenamide) produces a rubber having a lower degree of absorption than when dipentamethylenethiuram disulphide, tetramethylthiuram monosulphide, benzothiazyl disulphide, and tetramethylthiuram disulphide are used. These latter produce rubbers with a similar degree of absorption.

7. The inclusion of factice as a plasticiser causes a marked increase in the absorption.

8. Variation in the natural rubber used can result in a difference in the amount absorbed by the cured rubber mix.

In conclusion I wish to thank Messrs. J. C. Franklin & Son and particularly Dr. R. H. Muller for preparing and making available the special rubber mixes used in this investigation, also Dr. B. E. Tomlinson and Mr. G. B. Pendleton for the use of certain equipment.

## DISCUSSION

The papers were presented by the AUTHOR.

DR. G. E. FOSTER (Dartford) asked for more information about the physical condition of the samples of rubber used.

MR. R. L. STEPHENS (Brighton) said that the time of vulcanisation was important. Had the author determined the equilibrium constants using the same mix of rubber which had been given different vulcanising times?

MR. F. G. FARRELL (London) said the rate of absorption was also a function of the surface area of the rubber exposed to the solution and he asked the author to give the diameter and thickness of the rubber tubing used.

MR. D. H. MADDOCK (Dagenham) said that it had been reported that silicone plugs were very satisfactory for multidose containers. Had the author carried out any work with them?

MR. G. R. WILKINSON (London) asked whether the author had any information on the physical properties and suitability for use of the rubber after exposure to the chlorocresol.

DR. J. C. PARKINSON (Brighton) said it would be interesting to know if caps stored for 28 days until equilibrium was reached and then dried, absorbed more chlorocresol when they were used.

MR. T. D. WHITTET (London) asked if the other physical properties of the various samples of rubber tested had been examined.

MR. W. P. HUTCHINSON (Oxford) said it would be of interest to determine whether irradiation of rubber would render the surface impervious to chlorocresol.

MR. W. T. WING, in reply, said that the samples which he handled were cut from sheets approximately $\frac{1}{16}$ in. thick. The pieces were $\frac{1}{4}$ in. wide and 5 cm. long; this gave approximately 2 g. in each case. The rubbers prepared for his work were subjected to different heating times. In one case it was six minutes at 60 lb. pressure and in another twelve minutes, and in comparing the two there was little difference in the amount of absorption. Silicone caps were entirely unsatisfactory in that they were not sufficiently elastic and were not self-closing. He had investigated the physical properties of the rubbers which he had examined but had published no details. His main concern had been to examine chlorocresol and phenol absorption to try and find out the factors which affected it.

# AN INVESTIGATION INTO THE COMPACTION OF POWDERS*

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The mechanisms by which a mass of powder is compressed into a coherent tablet, compact or pellet are not yet fully understood. One subject requiring clarification is the knowledge of the transmission of forces through the compacting mass and of the effects of these forces within the mass. Several workers have touched on this problem[1–6] but the most useful contributions have been made from the field of powder metallurgy[7–12]. However, there is conflicting evidence about several aspects of the process[7–11], and it is possible that some of the theories which have been advanced to explain the mechanism of pressing, based on the behaviour of metals, will not necessarily hold when applied to non-metals. In the work described in this paper, compacts have been prepared from a suitable material under controlled conditions, partly in order to obtain additional evidence on the sequence of events in the compaction of powders and partly to provide information for use in determining operating conditions for further work[13].

## Experimental

### *Apparatus*

A horizontally split die was designed (Fig. 1), in which there were eight sections each of 2·5 cm. depth which permitted a maximum fill of 13·5 cm. An accurate location was achieved by an overlapping skirt having a taper of 3° on the lower edge of each segment. This engaged on a corresponding taper on the upper edge of the lower segment to give an exact fit. All segments were made of K.9 steel (Edgar Allen and Co. Ltd.) but were not hardened; they were located and clamped together to form a die by means of three studs screwed into the bottom segment. The upper punch, also made of K.9 steel, was a sliding fit in the die and had a diameter of 5·32 cm. and length 20 cm. The base plate and bottom punch were made of mild steel. A location slot was milled in the base plate in order to prevent relative movement between base, die and lower punch. This die-set was used between the platens of a 50-ton hydraulic press to give a maximum punch pressure of over 2000 kg./sq. cm.

### *The Powder*

Heavy Magnesium Carbonate B.P. was selected as the material most suitable for the pressings because it had constant powder characteristics of shape and size, it was free flowing, packed consistently and did not

* This account formed part of a thesis accepted for the degree of Ph.D. in the University of London.

cake during storage; it did not affect the materials of the die-set; it cohered when compressed, so that the resultant compact generally could be taken intact from the die for subsequent measurements; the particle size was negligible compared with the diameter of the die so that dimensional effects were reduced to a minimum. In order that different

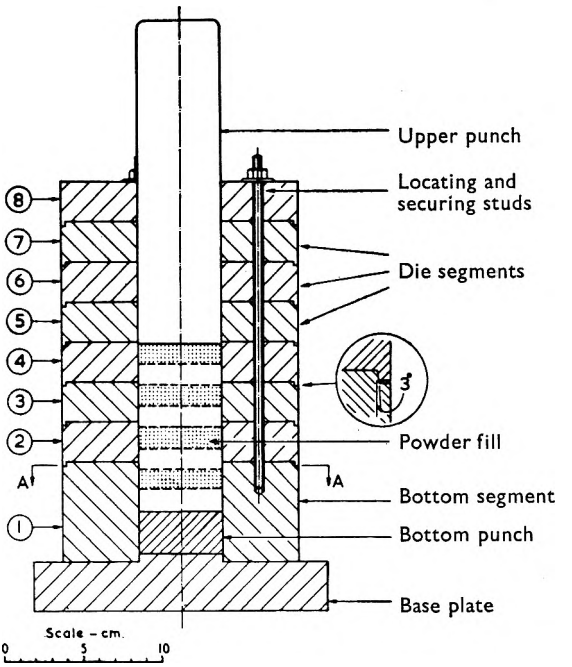


FIG. 1. Split-die assembly.

powder layers could be distinguished, 0·25 per cent. ammoniated carmine was used to produce a powder of satisfactory colouration to contrast with the natural white colour of the material. The particle size was standardised for the powder of both colours by using the 200–240 mesh fraction after sifting on an Inclyno sifter. The experiments were planned so that the powder fill could be made up of alternate layers of different colour.

*The Compacts*

The main factors affecting compaction are applied pressure, depth of powder and condition of die wall surface. A manageable experimental series to include all these factors was made to the following plan.

(*a*) *Three levels of pressure.* Final mean pressure of top punch, (i) 671 kg./sq. cm., (ii) 1358 kg./sq. cm., and (iii) 2040 kg./sq. cm.

(*b*) *Three depths of powder.* Controlled by weight and subsequent hand tamping to predetermined levels, (i) 80 g. in 20·0 g. layers (2 white, 2 pink), (ii) 160 g. in 20·0 g. layers (4 white, 4 pink), and (iii) 240 g. in 20·0 g. layers (6 white, 6 pink).

(*c*) *Two surface conditions.* (i) "unlubricated" plain wall, and

(ii) "lubricated" wall—lubricated with colloidal graphite (acetone "dag" (Acheson Colloids Ltd.)).

This gave 3 × 3 × 2 = 18 experiments for which a suitable experimental pattern was devised (see Table I).

TABLE I

EXPERIMENTAL PATTERN FOR PRESSING STUDIES

| Pressing | Compacting pressure (kg./sq. cm.) | Weight of powder fill (g.) | Type of surface |
|---|---|---|---|
| P.1 | 1358 | 80 | lubricated |
| 2 | 671 | 80 | unlubricated |
| 3 | 671 | 160 | unlubricated |
| 4 | 671 | 160 | lubricated |
| 5 | 1358 | 80 | unlubricated |
| 6 | 2040 | 160 | lubricated |
| 7 | 1358 | 240 | lubricated |
| 8 | 2040 | 80 | lubricated |
| 9 | 2040 | 240 | lubricated |
| 10 | 671 | 240 | unlubricated |
| 11 | 671 | 80 | lubricated |
| 12 | 1358 | 240 | unlubricated |
| 13 | 671 | 240 | lubricated |
| 14 | 1358 | 160 | lubricated |
| 15 | 2040 | 240 | unlubricated |
| 16 | 2040 | 80 | unlubricated |
| 17 | 2040 | 160 | unlubricated |
| 18 | 1358 | 160 | unlubricated |

*Method*

All surfaces to be in contact with the powder were degreased with equal parts acetone and carbon tetrachloride mixture and then polished with "Bluebell" metal polish; the internal surfaces of the die segments, including the part of segment No. 1 next to the bottom punch, were coated with a thin layer of colloidal graphite in those experiments employing lubrication.

The powder was poured into the die a layer at a time with frequent pauses for hand tamping to ensure a constant initial condition of packing of the particles. Each 20·0 g. layer was levelled to a predetermined height using a depth gauge. After the die had been assembled and the top punch slipped into position, pressure was applied with two minute intervals between increments after each of which the exposed punch length was measured. On reaching the required pressure, the load was released and measurements again taken of the exposed punch length and the height which the die had risen from the base plate. The die was taken off its base, placed on suitable supports and the compact forced out through its lower end, that is in the same direction as the applied pressure. Also, a note was made of the force required to produce the first detectable movement.

The height of the resulting compact was measured accurately and it was cut longitudinally using a fret-work machine; if the compact was broken or cracked, it was embedded in paraffin-wax before cutting. Photographs of some of the results are shown in Figure 2.

## RESULTS AND DISCUSSION

*The Sections*

An inspection of the longitudinal sections brings out several interesting features. The most striking observation is the differences in relative

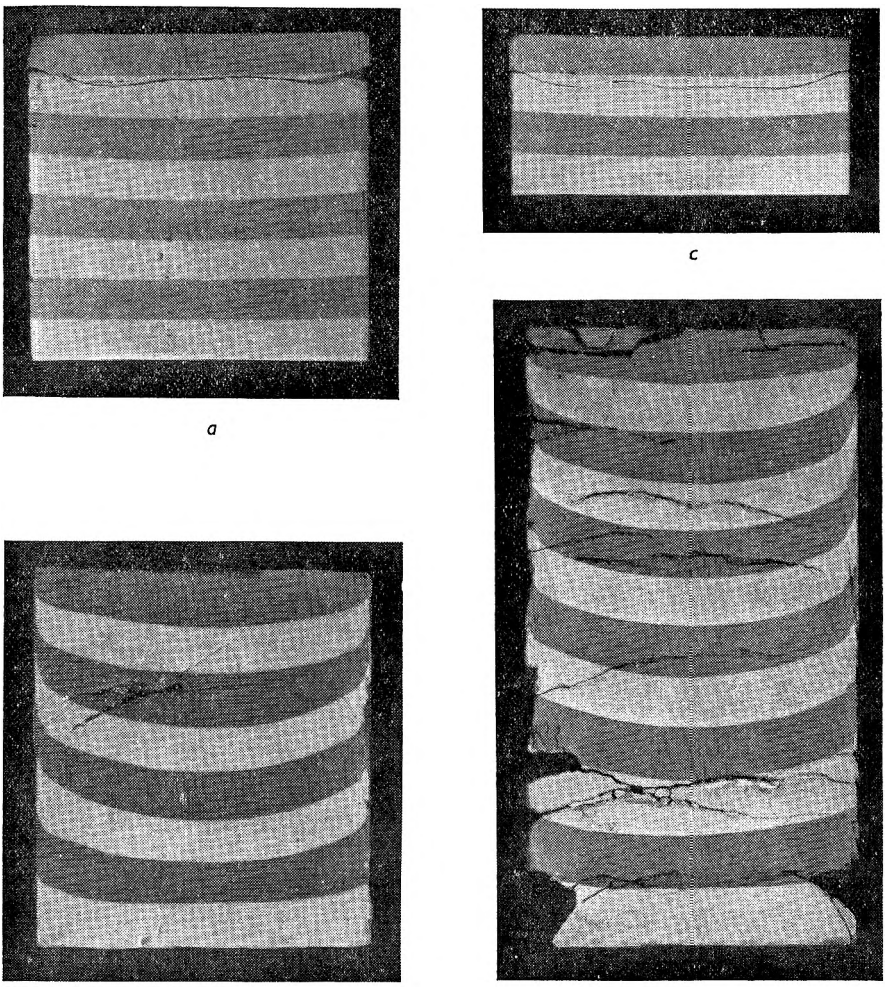

FIG. 2. Cut sections of compacts after ejection from die.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *a*. | Pressing, | p. 6, | compacting pressure | | 2040 | kg./sq. cm. | in | lubricated die |
| *b*. | ,, | p. 17, | ,, | ,, | 2040 | ,, | ,, | unlubricated die |
| *c*. | ,, | p. 8, | ,, | ,, | 2040 | ,, | ,, | lubricated die |
| *d*. | ,, | p. 12, | ,, | ,, | 1358 | ,, | ,, | unlubricated die |

displacement of the layers of material when compacted to the same level of pressure, but using different conditions of lubrication (Fig. 2*a* and *b*). This feature has been noted by various workers in the powder metallurgy field[7,8,14]. The differential displacement is caused by the greatly increased frictional forces occurring at the face of an unlubricated die wall, opposing the movement of the adjacent compact material which is tending to move as a result of the thrust from the top punch. On the other hand, the material at the centre is relatively free to move, being subject only to

normal interparticulate friction. Consequently the layers are unevenly displaced in the manner shown in the sections.

A second interesting observation is the presence in the compact of cracks of two types. The first can be seen as a split near the top of a compact (Fig. 2*a*). The shape is slightly concave in an upward direction along the axis of symmetry and roughly follows the line of demarcation between the top two layers of material. This type of crack is common in the lubricated pressings but is also found in the unlubricated pressings. The second type is concave in a downward direction and is to be seen in the sections of the unlubricated compacts which were subjected to the heavier pressures (Fig. 2*b* and *d*). How these cracks may arise is discussed later.

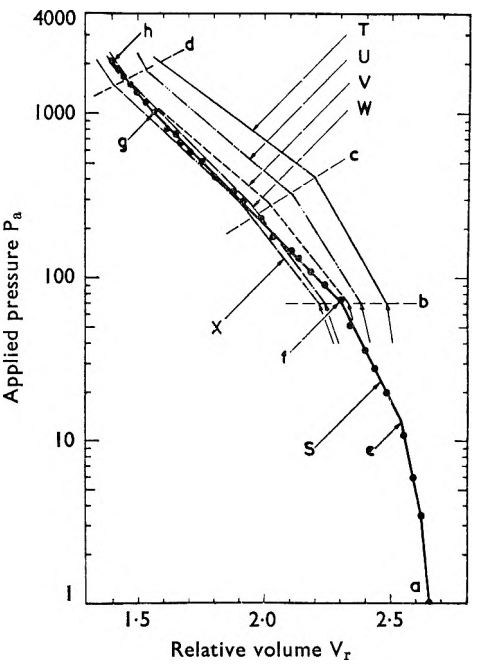


FIG. 3. Relation between applied pressure and relative volume of compact. In the case of curve S,

| | | | |
|---|---|---|---|
| Stage | I | extends from | a to e |
| ,, | II | ,, | ,, e to f |
| ,, | III | ,, | ,, f to g |
| ,, | IV | ,, | ,, g to h |

*Applied Pressure-Relative Volume Relationships*

From the measurements the exposed punch length taken during a pressing, the height of the compact within the die after each pressure increment was calculated, allowance being made for the shortening due to elastic compression of the top punch. Since a simple cylindrical shape was used, the relative volume $V_r$, defined by Walker[1] and used by Bal'shin[7] is given by dividing the observed length, L, by the length of the material when present as a true solid, $L_s$, i.e.,

$$V_r = \frac{L.\pi.D^2/4}{L_s.\pi.D^2/4} = \frac{L}{L_s}$$

where D is the diameter of the compact.

This ratio was computed for each height and the relations between the relative volume and the applied pressure $P_a$, for the 18 pressings are summarised in Figure 3 by lines T to X. T, U and V represent the results obtained when 12, 8 and 4 layers of powder are pressed in an unlubricated die, whilst W represents 12 and 8 layers, and X, 4 layers

of powder pressed in a lubricated die. For this series, the average relative volume of the powder fill was 2·66 ("a" in Fig. 3) and the first stage, represented by short arrows at the base of each curve, for each pressing was an applied pressure of 70 kg./sq. cm. This level has been shown as the horizontal line "b" and must be considered the datum line for this series, even though these compacts have a previous history of formation up to that pressure. It will be noted that each curve consists of three sections delineated by abrupt changes of slope. These breaks occur along roughly the same lines which have been labelled "b", "c" and "d".

Curve S is derived from a further series of 10 lubricated pressings in which readings were taken at a larger number of pressure levels. In this case a mean value of $V_r$ for each value of $P_a$ was obtained and the results plotted in Figure 3. There are eight readings below line "b", thus providing information for a region not included in the preliminary pressings.

It is possible to suggest an explanation of the changes in slope in the curves in terms of the changing physical condition of the material being pressed. It will be seen that such changes occur in curve S at points "e", "f" and "g". Stage I occurs in the region "a" to "e", i.e., up to 13 kg./sq. cm.; here decrease in the relative volume is most probably caused by interparticulate slippage of the powder, leading to closer packing. This process, however, is limited since the particles soon become immobile relative to one another and give rise to Stage II. This condition, found between "e" and "f", is characterised by the formation of temporary struts, columns and vaults[15] protecting small voids and generally supporting the imposed load. Stage III extends from "f" to "g", when the structure of the material fails either by crushing or by plastic flow. This takes place because there are point and line contacts between the asperities and angular edges of the particles, and, as $P_a$ increases, the stresses are transmitted from particle to particle through these contacts. Although the force transmitted between two particles may be relatively low, it takes place over such a small area that high stresses are imposed locally causing the material to fail. For the system under examination, this failure begins at about 70 kg./sq. cm., and extends to about 1000 kg./sq. cm.

However, since this failure takes place in a confined space, the freshly produced surfaces will be held in close proximity to one another under the influence of smaller transverse stresses. This is a favourable condition for bonding and "cold welding". Whether or not bonding actually does take place, depends on the coherent and adherent properties of the material. With magnesium carbonate, there is a strong coherent property; thus, although the powder is being broken down by the increasing load, simultaneously it will be rebonding to form a more closely packed structure. For a time the rate of breakdown will greatly exceed the rate of rebonding and this idea is supported by the work of Higuchi and others[3] who have shown that the specific surface area of a material increases during the initial stages of compaction. However, as the stresses increase,

a condition eventually develops, Stage IV, when the structure formed is strong enough to support the imposed load and any further reduction in volume of the compact involves the normal compressibility of the solid material. This stage is probably developing at point "g" on curve S and any permanent decrease in the voids of a still porous compact in this state can only be achieved either by exceeding the crushing strength of the structure or by plastic deformation, or both.

It must be emphasised, however, that this explanation is based on evidence obtained from external measurements and that as pressure is applied to a powder, various zones within the pressing will be subjected to different reliefs of pressure so that the particles in these zones will be at different stages of compaction. Thus the combined condition, given by external measurements, may not truly reflect the internal measurements.

In the preliminary pressings, due to the fact that the first pressure increment was 70 kg./sq. cm., the system was not able to accommodate itself for Stages I and II. Consequently in the next increment (to 110 kg./sq. cm.) imposed forces were being applied which could cause crushing and breakdown of powder near the top punch, whilst that near the bottom punch was still only being packed more closely. Thus the region "b" to "c" in Figure 3 is really a condition when there is a mixture of elastic and plastic deformation taking place and the result is a slope midway between that given by Stages II and III of curve S. It is not until the region between "c" and "d" is reached that the slope of the curves for the preliminary pressings approaches that of Stage III of curve S.

Observations of the compact state after compression serve to support the suggested explanation. The individual particles of powder of a pressing made at 28·5 kg./sq. cm. were only packed together and were easily removed from the surface of a cut cross-section using a camel hair brush; in a pressing made at 62 kg./sq. cm., the packing was firmer and the particles harder to dislodge; at 90 and 145 kg./sq. cm., there was a definite keying and bonding of the particles; at 336 and 671 kg./sq. cm., the compacts had become quite firm and the outline of the individual particles was difficult to trace even with the use of a low powered microscope; for pressings made to 1358 and 2040 kg./sq. cm., the compact to all appearances was a solid block, and, using a microscope, only occasionally could the outlines of the original particles be seen.

There is supporting evidence for this explanation in published work. Various descriptions of the compaction sequence have been noted[7,8,16,17], but as far as can be ascertained, only Huffine[17] has attempted to trace to any extent the various stages using the relation between applied pressure, $P_a$, and the relative volume, $V_r$. The author considers that probably too much attention has been directed to the exponential relation which usually exists at some stage of the process, and as a result evidence for the existence of other relations was missed.

A further experimental point is that at all stages a "skin" was produced where the compacts had been in contact with the die-walls, and the particles in this region were always relatively much more distorted and bonded together compared with those situated within the compact. This

condition is attributed to the local shear forces produced during compaction and extrusion causing breakdown with simultaneous rebonding of particles to give a denser structure of skin thickness only. In compacts pressed above 145 kg./sq. cm. the particles in contact with the punches appear to be bonded together more strongly than the internal particles adjacent to them. This may be explained by the development of a special boundary condition, where the particles are being crushed against a hard surface, the fragments filling up the voids and rebonding. An analysis of the forces which cause crushing under these conditions was first made by Hertz[18]. It must be emphasised that the bonding near the punch surfaces was never so marked as that at the die walls.

*Elastic Recovery of the Compacts*

There is a stage at which the powder has formed a structure which, although still porous, is strong enough to support the applied pressure and the natural elastic properties of the material become important as is shown by the fact that the relative volume exhibits a recovery when the pressure is released, especially with the lubricated compacts (Table II).

TABLE II

RECOVERY IN LENGTH AFTER RELEASE OF APPLIED PRESSURE

| Compact size g. | Lubricated die | | Unlubricated die | |
|---|---|---|---|---|
| | Per cent. recovery on release of $P_a$ | Per cent. recovery when extruded | Per cent. recovery on release of $P_a$ | Per cent. recovery when extruded |
| 80 | 2·3 | 4·4 | 2·5 | 4·2 |
| 160 | 3·3 | 4·8 | 1·6 | 4·6 |
| 240 | 2·5 | 4·2 | 1·0 | Not measurable |

Further increase in volume takes place as the compact is ejected from the die when the total recovery is the same for all compacting conditions of this series and seems independent of the condition of the wall surface. It would appear that elastic recovery takes place only to a limited extent within the die, frictional forces on the walls opposing complete relaxation especially in the unlubricated die, and full recovery is possible only after the compact has been ejected.

*Force Required to Produce First Movement*

Inspection of the values for the force, $F$, in units kg. weight, required to produce first movement of a completed compact in a die shows two trends. The first is that there is an exponential relation between $F$ and the applied pressure, $P_a$, provided all other conditions are kept constant. The second is that $F$ is related exponentially to the height, $L$, and therefore to the final wall surface, $S$, of the compact, all other conditions being kept constant. It was found advantageous to combine $P_a$ with $S$ as a product and the logarithm of this function (log. $P_a.S$) was plotted against log $F$ as shown in Figure 4. Results of 24 other pressings using lubricated die-walls and a wide range of pressure conditions have also been included in this figure.

There are two sets of results according to the condition of the die surface. For the unlubricated die, the results fall about a "best straight line", line A, which fits the equation:

$$\log F = 1{\cdot}43\ (\log P_a.S) - 2{\cdot}68$$

i.e.,
$$F = 0{\cdot}0021(P_a.S)^{1.43} \quad .. \quad .. \quad .. \quad ..(1)$$

In the case of the lubricated die, the line fitting the points is B, the equation of which is:

$$\log F = 1{\cdot}43\ (\log P_a.S) - 3{\cdot}39$$

i.e.,
$$F = 0{\cdot}00041\ (P_a.S)^{1.43} \quad .. \quad .. \quad .. \quad ..(2)$$

Conclusions which may be drawn from this correlation can be only tentative, but two points are worthy of comment. The first is that the equations derived from the graph are dimensionally homogenous, which indicates that the correlation may have a physical basis. The second is that it is possible to develop a theoretical approach to aid the understanding of the correlation and of the mechanism associated with the frictional effects developed during compaction and ejection.

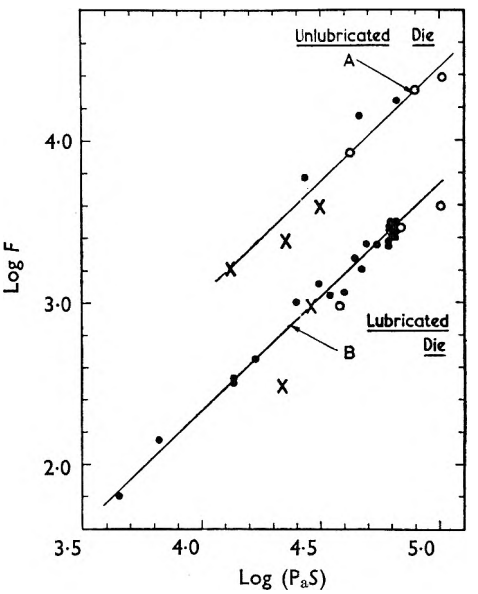


FIG. 4. Relation between the force required to produce first movement, $F$, and the product of maximum applied pressure, $P_a$ and the final surfaces $S$.

× 80 g. compacts
● 160 g. ,,
○ 240 g. ,,

If the compacting system is analysed at the time at which the maximum applied pressure has been reached, it will be seen, Figure 5, that this pressure, $P_a$, will be transmitted to the material immediately below the top punch. Let the mean vertical stress on a horizontal plane at distance, z, from the top punch face be defined as $P_z$: then it is reasonable to assume that

$$P_z = f_1(P_a,z),$$

and that the nature of the function, $f_1(P_a,z)$ will be the same for similar conditions of pressing.

If the radial stress along the horizontal plane, distance, z, from the datum surface and radius, r, from the axis of symmetry, is $P_{r(z)}$ there will be a relation between $P_z$ and $P_{r(z)}$ depending on the physical properties of the material and possibly on the condition of the die-wall surface.

If these conditions are held constant for a particular experimental series, then we may write

$$P_{r(z)} = f_2(P_z,r),$$

and it follows

$$P_{r(z)} = f_3(P_a,z,r).$$

Let $d(F_z)$ be the frictional force at the die wall tending to prevent motion of the element of thickness, $dz$, and which is subject to the radial stress, $P_{R(z)}$ at the wall surface of the compact of radius, R: then,

$$d(F_z) = \mu_z P_{R(z)} \pi 2R\, dz$$
$$= \mu_z f_3(P_a,z,R)\pi 2R\, dz,$$

where $\mu_z$ is the coefficient of friction between the compact and the die-wall at z.

Over the whole wall surface of the compact the force, F, resisting movement will be

$$F = \int d(F_z) = \int_0^L \mu_z f_3(P_a,z,R)\pi 2R\, dz, \quad \ldots \quad \ldots (3)$$

where L is the length of the compact under the conditions considered.

Equation (3) is a general equation for the system and can only be solved when the relation between the various factors are known. This requires a complete knowledge of the mode of stress transmission within the material and a knowledge of the change in value of the coefficient of friction, $\mu_z$, with z. The former information is not known at present and the variation in the coefficient of friction along the length of a compact has only recently been demonstrated. Thus Unckel[14] in testing his theory of stress distribution within a compact used a coefficient of 0·2, whereas Kamm, Steinberg and Wulff[9] found in some of their pressings that the value of $\mu_z$ varied from 0·625 near the top punch to less than 0·07 near the bottom.

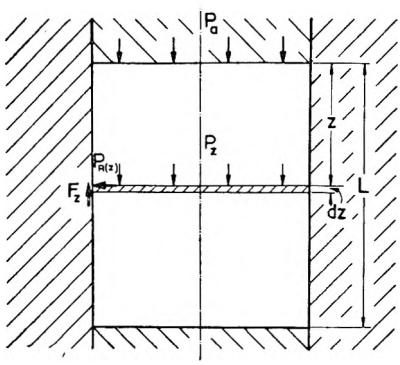


FIG. 5. Forces acting in a compact before maximum applied pressure, $P_a$, is released.

Before extrusion can take place the applied pressure is released and the material exhibits an elastic relaxation to which attention has already been drawn in this discussion. Residual stresses remain in the compact and certain other stresses are reintroduced when the force, $F$ necessary to produce first movement for extrusion is applied. This gives rise

to a new set of stresses acting in the compact and it is a function of these stresses, distinguished in the following formulæ by the index $^e$, which combined with the coefficient of friction, $\mu_z^e$, acting on the walls, tends to oppose motion when the extrusion process takes place.

$$\text{That is, } F = \int d(F_z) = \int_0^L \mu_z^e f_4(P_z^e, R)\pi\, 2R\, dz \quad .. \quad ..(4)$$

It is probable that $\mu_z = \mu_z^e$

and also that

$f_4(P_z^e R)$ is simply related to $f_3(P_a,z,R)$.

This is supported by evidence obtained from a paper by Nelson and others[4] who published data (Table III in their paper) on the forces developed during the production of tablets of 0·954 cm. diameter, using compacting forces of up to 1300 kg. weight. All experimental conditions were kept constant, except the amount and type of lubricant. From the information supplied, it was possible to calculate the force, F, resisting movement at the die wall at maximum applied pressure, and also the force, *F*, to produce first movement for ejection, and, as shown in Figure 6, there appears to be a simple relation between F and *F*. This graph means that, if $\mu_z$ is proportional to $\mu_z^e$, then $f_4(P_z^e,R)$ must be of the same form as $f_3(P_a,z,R)$.

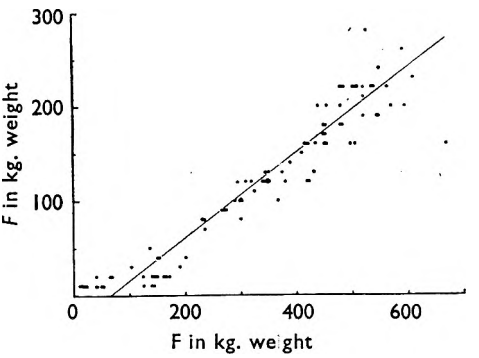


FIG. 6. Relation between the force, F, resisting movement at the die wall at maximum applied pressure and the force, *F*, to produce first movement for ejection (From Nelson and others[4]).

The results of the present work point to a relationship of the form

$$F = c(P_a S)^m \quad .. \quad .. \quad .. \quad .. \quad .. \quad ..(5)$$

This equation is consistent with the analysis above. The constant $m$ is a property of the material used and possibly of the physical dimensions of the system, whereas $c$ is a property of the surface condition of the walls. It is probable that in making other series of pressings of the same nature, but varying the lubricant on the walls, other lines parallel to A and B in Figure 4 would be obtained. For example, if a good lubricant such as stearic acid were used, the resulting line would lie near that of line B and if a material like colophony resin were used to coat the walls the line would be parallel to and lie near line A.

*Apparent Density Calculations from Sections*

By reason of symmetry, the rotational movement of particles in an evenly packed cylindrical powder mass has no significance. Several

workers[7,9,14,19] have stated that there is little evidence to indicate radial movement in a powder mass when an even compacting force is applied in a die with well lubricated walls. It follows that the only significant movement that the material can make is along the z-axis. That being so, if a given weight of material is evenly distributed horizontally in layers before pressing, it will be possible to calculate the degree of compaction in various regions of the resulting compact from measurements of the vertical heights of the appropriate layers seen in a half section of the compact.

The cut sections of the lubricated compacts were inspected and the heights of the various layers measured using a Universal measuring machine (Cambridge Instrument Co.); the verticals chosen were the centre-line, together with lines parallel to and at a distance from it of R/4, R/2, 3R/4, 7R/8 and R where R is the radius. These latter readings were taken on both sides of the centre-line and a mean reading recorded. From the measurements the per cent. solid present was calculated and these figures were transferred to an outline of the compact; lines of equal apparent density were then drawn in the three diagrams on the left of Figure 7. Evidence confirming the validity of this procedure will be presented in another paper[13].

For sections of compacts made with unlubricated walls, the assumption that no significant radial movement takes place is not necessarily true. Because of this any apparent densities determined by the above procedure can be used only as a guide or a first approximation and cannot be considered to be correct until proved by some other means. However, it was of interest to see what picture would emerge if the method were used on some of the sections to hand, so a group of 8-layered "unlubricated" compacts was measured and the results are presented in the right-hand column of diagrams in Figure 7.

Inspecting the results of the lubricated compacts first (Fig. 7*a*, *b* and *c*), it can be seen that there is a region of greater apparent density in the top corners of all pressings, and a region of lower apparent density in the bottom corners. The greatest difference between the figures closest to the wall (line 7R/8) is only about 5 per cent., but those on the surface show up to 11·5 per cent. In the body of the pressings, there is a region of lower density near the top centre and a region of higher density about two-thirds of the way down. The greatest difference amounts to 6 per cent. The interesting feature is that the region of higher density on the centre-line is roughly of the same magnitude as that in the top corners and these regions are connected by "ridges" exhibiting a lower density. In the more heavily pressed compacts, *b* and *c*, the low density region in the centre is about the same apparent density as that in the bottom corners. In general these diagrams are similar to the examples given by Kamm, and others[9].

A similar picture is seen in the results of the unlubricated pressings but it may be distorted due to undetected radial movement. Here, the range in densities is greater and the higher density region on the centre-line has not the same magnitude as that found in the top corners. When

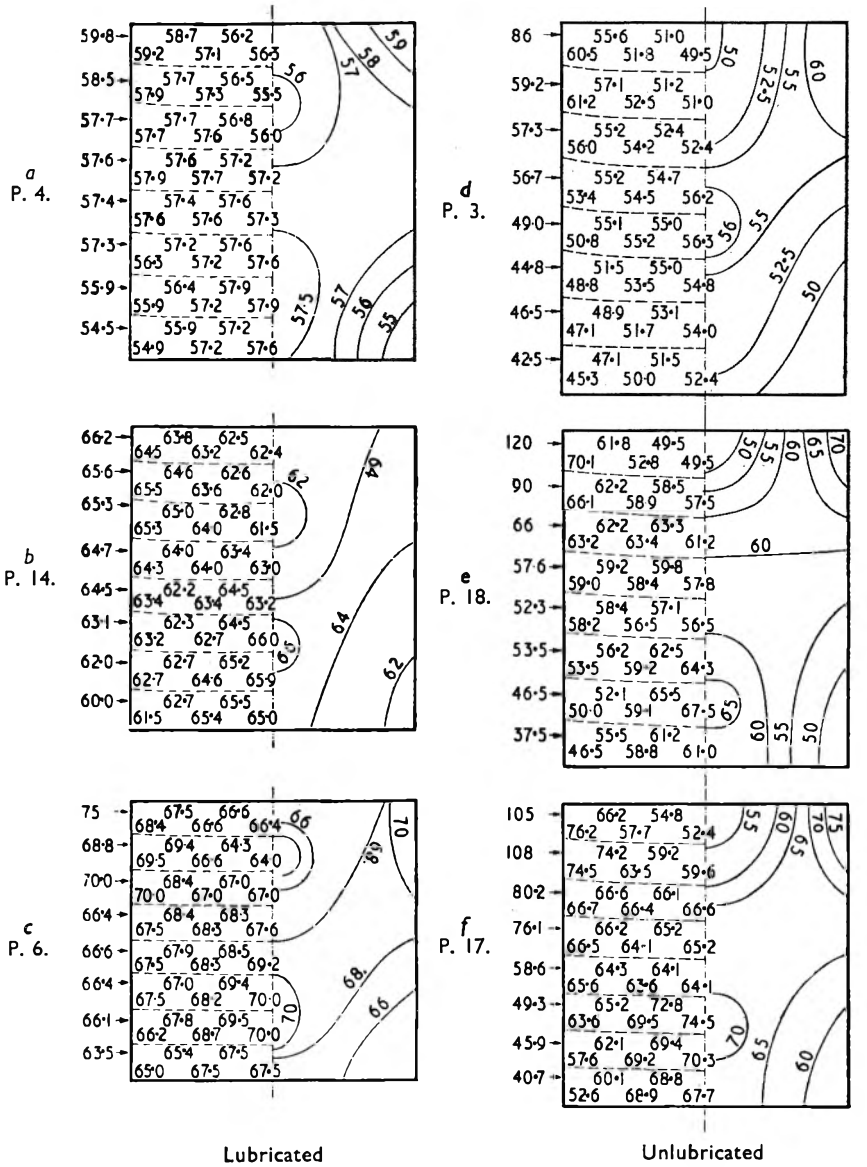


FIG. 7. Apparent density distributions in preliminary pressing expressed as per cent. solid present.

compared with densities just within the pressing, those on the edge show sufficient discrepancy to indicate that movement of material takes place during extrusion, causing a concentration towards the top end. This shows particularly in the unlubricated compacts where apparent densities of "120" per cent. would appear to have been achieved. For

this reason the surface figures have not been taken into account when drawing the contours.

There is an interesting point about the region of higher density found in the lower centre portion of the compacts. The presence of a denser structure such as this in a normal tablet could explain the well-known empirical observation in a disintegration test where the outer layers of the tablet disintegrate and slough away quite rapidly leaving a core which requires a longer period before it breaks up.

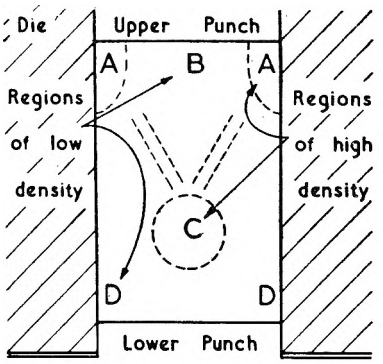


FIG. 8. Conditions in compact before applied pressure is released.

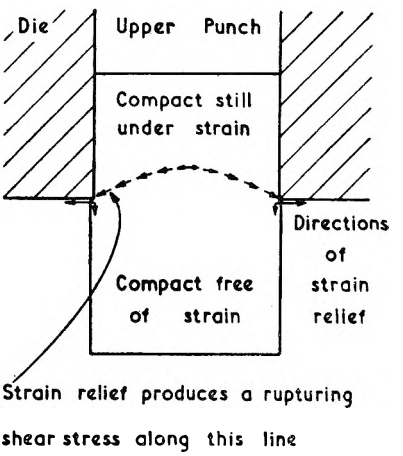


FIG. 9. Extrusion of unlubricated compact.

The variations in apparent density that exist in different regions of a compact have been noted. Evidence of and comment on the formation of these regions will be given elsewhere[13], but their effect on the subsequent history of the compact will be discussed now. On the release of the maximum applied pressure, there will be a peripheral elastic relaxation along the longitudinal axis in zone A (Fig. 8) and this will induce a longitudinal stress in B, where the material is less dense and therefore probably weaker. Since the structure of the compact is such that it is not strong in tension, a relief of weakness will be produced. Any additional stress due to some other factor will tend to cause a laminar crack to develop through the material. Attention has already been drawn to cracks of this form in pressings made for this work, and it is probable that this condition is present to a greater or lesser extent in all compacts. It is suggested that this is the basic cause of the phenomenon called "capping". Capping is that condition where the top of the pellet or tablet "becomes detached and even when this is not immediately apparent, the 'cap' can be removed with the thumb nail or it will fall off when a few tablets are shaken in a bottle"[20]. The reasons for capping are various, and amongst those listed by Silver and Clarkson[21] are (*a*) too much pressure, (*b*) entrapped air, and (*c*) insufficient binder. In each

case the "reason" is the obvious or visible factor which is sufficient to increase the stress at the capping site to breaking point.

The relaxation of the elastic strains in the lower part of the pressing (C in Fig. 8) takes place in a downward direction causing the die-block to be lifted off the base plate. Induced tensile stresses are probably developed in the peripheral parts, D, but, since there are no cracks in the lubricated pressings in this region, the breaking or cracking stress is probably not exceeded. The stress pattern in the case of the unlubricated pressings is very complex even after the maximum applied pressure has been released. Since the elastic strains are only partially relieved at this stage (Table II), full relaxation can only take place on extrusion. Further, even during extrusion, the stresses induced by friction at the walls hold the strained condition in the material until the edge of the die is reached when a spontaneous expansion in two directions takes place (Fig. 9). Thus the material outside the die is relaxed whereas that inside the die is still stressed. The imposed strain exceeds the shear strength of the material resulting in the series of cracks to which attention has already been drawn in Figure 2.

## Summary

1. Using Heavy Magnesium Carbonate B.P., the differential movement of a powder has been investigated in a cylindrical die when subjected to applied pressures of up to 2000 kg./sq. cm. from one end.

2. The relation between the relative volume of the material and the applied pressure has been determined for selected conditions of pressing, and a possible explanation in terms of the changing physical condition of the material is advanced for the changes in slope which occur in the curves.

3. Studies have been made correlating the force to produce first movement for extrusion, the maximum applied pressure and the final wall surface of the compact.

4. The variations in relative density within a compact have been estimated and a tentative explanation has been made of the cause of the phenomenon known as "capping".

The author wishes to thank Professor D. M. Newitt, F.R.S. for his advice and interest in this work and for providing research facilities in his Department.

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## DISCUSSION

The paper was presented by the AUTHOR.

DR. F. HARTLEY (London) enquired why the author had selected a basic substance, heavy magnesium carbonate, as the material for his work. Was the same batch used throughout and was the moisture content uniform?

MR. E. W. RICHARD (Upminster) said the author had used material and procedures which were far removed from the normal conditions of pharmaceutical tablet manufacture. The die walls and punch faces were lubricated, whereas in tablet manufacturing practice the granules were lubricated. One could foresee that that would make a considerable difference to the internal stresses set up during compression. Then, again, pressure was applied at intervals of two minutes, and the increments of pressure were not stated. Flat punches were used, but the internal stresses in a biconvex tablet would be very different: also contrary to normal procedure, the compact was extruded from the die in the same direction as that of the applied pressure. It was not clear whether the author was aware of the difference between capping and lamination. By "capping" was meant the phenomenon whereby the surface layer of the tablet broke away when in a certain form, and by "lamination" was meant the horizontal splitting open of a tablet. In his experience he had found those two conditions to be quite distinct.

MR. H. BURLINSON (Ashton-under-Lyne) said inorganic powders did not present the same problems in compression as organic ones. Had the author any experience of using organic compounds?

MR. A. AXON (Dartford) asked how the ammoniated carmine was incorporated. Could some information be given about the shape and size of the particles, and whether there was any aggregation. It was not clear from the paper why the author had used a horizontally split die. It would seem that in the method of compaction the uppermost layer would be the least compacted. He wondered whether it was significant that the split near the top of the compact occurred between the two least compacted layers in that region. It was also difficult to appreciate why there were the marked changes in slope of curve S in Figure 3.

DR. D. TRAIN, in reply, said he had chosen heavy magnesium carbonate because he wanted a material which was easy to handle. He had extensively reviewed the literature, and had come to the conclusion that

for this initial work all he needed was a simple, readily available substance. A sample of 200 to 240 mesh powder was selected, dried for 24 hours at 110° C., allowed to humidify for 2 hours under standard conditions and then 80 g. quantities were placed in sealed containers. The containers were opened just before pressing was carried out; the moisture content was about 0·5 per cent. under those conditions. He had been taken to task for departing from the normal procedure of tablet manufacture but he had not intended to follow this. He had chosen a cylinder because it was the simplest practical shape on which to make measurements, he wanted a size which would facilitate manipulation of the powder fill, so he chose a die of 5 cm. diameter, and because he found difficulty in packing the powder fill in horizontal layers, he had split the die horizontally so that it could be built up as it was filled. He felt that the rôle of lubricants in the compaction process needed clarifying. The pressures were applied in roughly a logarithmic increment. Over the total range he had approximately 30 pressure levels. So far as extrusion in the same direction was concerned, again he did not want to complicate a simple picture. He wanted to find out what the compact looked like when pressing was continued in the same direction and he felt that by pressing in the opposite direction the contour of the layers within the compact would be altered because the direction of applied force had been reversed. Ammoniated carmine was used because it was simply made and easily incorporated. The pink and white powders under microscopic examination, appeared to be identical in shape and the constituent particles were not aggregated and had a shape factor of about 80 per cent. sphericity. It would be seen from Figure 7 that the uppermost layer of the compact showed quite a difference in its final condition. There was a wide difference in the apparent density distribution across a horizontal line near the top. It could not be said that any one of the layers was less compacted before compression because they were all the same. Each layer was hand tamped to a predetermined level so that every compact consisted of layers of powder with the same distance between them. With regard to the question of rigid stages, although line S (Fig. 3) represented the mean of approximately 15 pressings and each point had been put in separately, the relation between the series of points in each experimental run was so characteristic and reproducible that it was felt that attention should be drawn to the sharp changes of slope.

# THE RHEOLOGY OF OIL-IN-WATER EMULSIONS

## I. The Effect of Concentration of Constituents on Emulsion Consistency

By Arnold Axon

*From the Wellcome Chemical Works, Dartford, Kent*

Received June 21, 1956

In a previous paper on the consistency of oil-in-water emulsions[1] it was stressed that semi-solid preparations show different kinds of anomalous viscous behaviour, and that to distinguish between them a complete consistency curve must be determined. Suitable consistency curves could be obtained with the variable speed rotational viscometer originally described by Green[2]. Oil-in-water emulsions of liquid paraffin and Emulsifying Wax B.P., both with and without bentonite were examined. Most exhibited shear-rate thinning, as might have been expected from the observations of many workers since Hatschek[3] first showed in 1911 that the viscosity of an emulsion varied with the rate of shear. The emulsions containing bentonite when autoclaved exhibited uniform plastic flow, an observation not previously reported. This has provided a basis for the comparison of this kind of emulsion, since uniform plastic flow can be characterised by two values, "U", the plastic viscosity in poises, and "f", the yield value, in dynes per sq. cm.

This paper records the preparation of oil-in-water emulsions of liquid paraffin, cetyl alcohol, sodium lauryl sulphate and bentonite and describes the effect on the consistency of changes in the concentration of each constituent of the emulsion. The work is part of a larger project which aims to show in quantitative units the significance of such expressive terms as "body", "podgy", "sloppy", and "stiff" which are incapable of conveying an accurate description of these physical characteristics. From a physico-chemical viewpoint, emulsions are complex systems and many variables are known to affect their ultimate viscous properties. A review of the literature on the consistency of emulsions gives an indistinct picture of the effect of the concentration of the constituents. This appears to be due to the difficulty of comparing emulsions which show shear-rate thinning[4] and the inadequacy of empirical methods of measurement for comparison purposes[5].

### *The Effect of the Concentration of the Emulsifying Agent*

Several workers[6–10] have shown that different emulsifying agents will yield emulsions of markedly different consistencies for the same phase concentrations, but the effect of varying the concentration of the emulsifying agent has had few reports. Wilson and Parke[11] reported that the viscosity in a U tube viscometer, of mobile oil-in-water emulsions containing 70 per cent. of disperse phase increased with the concentration of the emulsifying agent. Toms[6] also showed, in a series of oil-in-water

emulsions of equal phase ratio prepared from a variety of organic liquids as internal phase and a number of univalent soaps as emulsifiers, that an increase in the soap concentration produced in most cases an increase in viscosity, the magnitude of which varied with the nature of both the internal phase and the soap.

*The Effect of the Volume Concentration of the Disperse Phase*

In a variety of emulsions, an increase in the concentration of disperse phase up to a critical value causes only a slight increase in the viscosity[8,12–15]. Above this critical concentration there is a sudden rise to a maximum viscosity above which instability and inversion of the emulsion occurs. Leighton, Leviton and Williams[16], showed a maximum value followed by a minimum in the curve relating viscosity and fat concentration in ice-creams when measured by the sagging beam method.

*The Effect of the Globule Size Distribution of the Disperse Phase*

There are conflicting reports on the effect of the globule size distribution on the consistency of oil-in-water emulsions. Sibree[17] reported that the limiting viscosities of fine oil-in-water emulsions of liquid paraffin emulsified with 1 per cent. sodium oleate solution were "not very different from that of the coarse emulsions". Leviton and Leighton[18] found no change in viscosity when the globule diameter was reduced from 3$\mu$ to 0·7$\mu$ in emulsions of 10 and 30 per cent. milk fat. Lyttleton and Traxler[19] and Terry, Gabriel and Blott[20], however, working on asphaltic bitumen emulsions, showed that an emulsion of equal sized globules was more viscous than an emulsion having a large variation in globule size.

## Experimental

*Preparation of Emulsions*

Emulsions were prepared to the following formulæ.

| | | |
|---|---|---|
| Liquid paraffin B.P. | 15 to 40 | g. |
| Sodium lauryl sulphate B.P. | 0·2 to 1·0 | ,, |
| Cetyl alcohol | 2 to 10 | ,, |
| Bentonite B.P. | 0 to 3 | ,, |
| Distilled water B.P. | 100 | ,, |

The cetyl alcohol was melted in the liquid paraffin by heating in a hot air oven at 70° C. The sodium lauryl sulphate was dispersed in this oil phase. Sufficient water at 70° C. was added to make the total weight 70 g. The emulsion was then formed by homogenisation with an immersion type homogeniser[21] for one minute. The emulsion after cooling to room temperature, was distributed in several pots and each was diluted with water or a bentonite suspension to give concentrations of bentonite from 0 to 3 per cent. in the finished product and mixed intimately. These final emulsions were then transferred to screw-capped jars and sealed with efficient white rubber wad closures.

## TABLE I

THE EFFECT OF CONCENTRATION OF CONSTITUENTS ON THE CONSISTENCY OF AUTOCLAVED OIL-IN-WATER EMULSIONS

| Constituent held constant Percentage w/w | Principal constituent varied Percentage w/w | Subsidiary constituents varied Percentage w/w | Effect on the limiting value of the viscosity at 25° C. Determined at $91{\cdot}4^{-1}$ sec. (200 r.p.m.) | Effect on the value of the torque intercept or yield value at 25° C. Determined at $91{\cdot}4^{-1}$ sec. (200 r.p.m.) |
|---|---|---|---|---|
| Liquid paraffin 25 | Bentonite 0 to 3 | Sodium lauryl sulphate 0·2 to 1·0<br>Cetyl alcohol 2 to 10 | Directly proportional to the bentonite content<br>Figure 1. Table II | Directly proportional up to 2 per cent. bentonite, but tends to increase exponentially above 2 per cent. bentonite at the higher concentrations of sodium lauryl sulphate and cetyl alcohol<br>Figure 2. Table II |
| Liquid paraffin 25 | Sodium lauryl sulphate 0·2 to 1·0 | Bentonite 0 to 3<br>Cetyl alcohol 2 to 10 | Directly proportional to the sodium lauryl sulphate content<br>Table II | Directly proportional to the sodium lauryl sulphate content at 2, 4 and 6 per cent. cetyl alcohol. Directly proportional above 0·4 per cent. sodium lauryl sulphate at 8 and 10 per cent. cetyl alcohol<br>Table II |
| Liquid paraffin 25 | Cetyl alcohol 2 to 10 | Sodium lauryl sulphate 0·2 to 1·0<br>Bentonite 0 to 3 | Increases exponentially with the cetyl alcohol content<br>Figure 3. Table II | Increases exponentially with the cetyl alcohol content<br>Figure 4. Table II |
| Cetyl alcohol 6 | Bentonite 0 to 3 | Sodium lauryl sulphate 0·2 to 0·8<br>Liquid paraffin 15 to 40 | Directly proportional to the bentonite content<br>Table III | Directly proportional to the bentonite content<br>Table III |
| Cetyl alcohol 6 | Sodium lauryl sulphate 0·2 to 0·8 | Bentonite 0 to 3<br>Liquid paraffin 15 to 40 | Directly proportional to the sodium lauryl sulphate content<br>Table III | Directly proportional to the sodium lauryl sulphate content<br>Table III |
| Cetyl alcohol 6 | Liquid paraffin 15 to 40 | Sodium lauryl sulphate 0·2 to 0·8<br>Bentonite 0 to 3 | Directly proportional to the liquid paraffin content<br>Figure 5. Table III | Increases exponentially with the liquid paraffin content<br>Figure 6. Table III |

TABLE II

AUTOCLAVED EMULSIONS CONTAINING 25 PER CENT. LIQUID PARAFFIN AND VARYING AMOUNTS OF SODIUM LAURYL SULPHATE AND BENTONITE

| Sodium lauryl sulphate per cent. | 0·2 | | 0·4 | | 0·6 | | 0·8 | | 1·0 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $U_{200}$* | $f_{200}$† | $U_{200}$ | $f_{200}$ | $U_{200}$ | $f_{200}$ | $U_{200}$ | $f_{200}$ | $U_{200}$ | $f_{200}$ |
| Bentonite per cent. | Emulsions containing 10 per cent. cetyl alcohol | | | | | | | | | |
| 0 | 5·86 | 1036 | 5·97 | 1665 | 6·36 | 1717 | 6·56 | 1834 | 6·67 | 1912 |
| 1 | 6·26 | 1178 | 6·49 | 1706 | 7·10 | 2185 | 7·41 | 2263 | 7·73 | 2361 |
| 2 | 6·93 | 1110 | 8·05 | 1990 | 8·47 | 2419 | 8·90 | 2458 | 9·53 | 2692 |
| 3 | 8·05 | 1581 | 9·06 | 2153 | 9·22 | 2848 | 9·32 | 3278 | 9·96 | 3844 |
| | Emulsions containing 8 per cent. cetyl alcohol | | | | | | | | | |
| 0 | 4·05 | 799 | 4·05 | 975 | 4·72 | 1218 | 4·42 | 1205 | 4·79 | 1259 |
| 1 | 4·17 | 812 | 4·94 | 1178 | 5·09 | 1286 | 5·09 | 1354 | 5·16 | 1571 |
| 2 | 5·01 | 907 | 5·60 | 1367 | 5·75 | 1598 | 6·34 | 1706 | 6·04 | 1923 |
| 3 | 5·68 | 1137 | 6·19 | 1760 | 7·00 | 1841 | 7·00 | 2029 | 7·20 | 2966 |
| | Emulsions containing 6 per cent. cetyl alcohol | | | | | | | | | |
| 0 | 2·96 | 415 | 3·11 | 493 | 3·01 | 549 | 3·01 | 618 | 3·29 | 793 |
| 1 | 3·01 | 337 | 3·04 | 484 | 3·46 | 636 | 3·46 | 751 | 3·76 | 948 |
| 2 | 3·54 | 542 | 4·05 | 812 | 4·13 | 894 | 4·27 | 1083 | 4·42 | 1462 |
| 3 | 4·13 | 691 | 4·42 | 1029 | 4·72 | 1164 | 4·57 | 1340 | 4·72 | 1503 |
| | Emulsions containing 4 per cent. cetyl alcohol | | | | | | | | | |
| 0 | 1·49 | 184 | 1·52 | 228 | 1·61 | 240 | 1·68 | 249 | 1·61 | 256 |
| 1 | 1·78 | 203 | 1·78 | 256 | 1·76 | 260 | 1·91 | 300 | 2·16 | 350 |
| 2 | 2·08 | 268 | 2·11 | 309 | 2·31 | 355 | 2·61 | 429 | 2·56 | 438 |
| 3 | 2·58 | 387 | 2·58 | 415 | 2·84 | 558 | 2·78 | 544 | 2·96 | 609 |
| | Emulsions containing 2 per cent. cetyl alcohol | | | | | | | | | |
| 0 | 0·83 | 86 | 0·85 | 88 | 1·05 | 124 | 1·11 | 129 | 1·14 | 171 |
| 1 | 1·04 | 92 | 1·14 | 118 | 1·21 | 131 | 1·28 | 157 | 1·19 | 191 |
| 2 | 1·18 | 129 | 1·28 | 157 | 1·46 | 187 | 1·62 | 194 | 1·76 | 230 |
| 3 | 1·35 | 157 | 1·66 | 212 | 1·77 | 233 | 1·87 | 235 | 1·88 | 237 |

* $U_{200}$ .. The plastic viscosity "U" in poises determined at 200 r.p.m. ($91{\cdot}4^{-1}$ sec.)
† $f_{200}$ .. The yield value "f" in dynes per sq. cm., determined at 200 r.p.m. ($9{\cdot}^{-1}$ sec.)

*Subsequent Treatment of Emulsions*

The jars of the different emulsions were subjected to two different heat treatments in order to make uniform the air content and the rate of cooling, and to study the changes in consistency due to autoclaving.

*Heating at* 70° *C.* The jars of emulsion were immersed for 30 minutes in a water bath thermostatically controlled at 70° C.

*Heating at* 115° *C.* The jars of emulsion were autoclaved at 115° C. for 30 minutes.

Narayanaswamy and Watson[22] and Sibree[17] have stated that the incorporation of small amounts of air can give misleading values for the consistency of an emulsion. Between 2 and 10 ml. of air per 100 g. was found to be incorporated as minute air bubbles in the more viscous emulsions during their preparation. All, however, were sufficiently fluid at 70° C. to enable the air bubbles to rise, yielding emulsions of negligible air content, shown by tests on some by the method described by Blagg[23].

TABLE III

AUTOCLAVED EMULSIONS CONTAINING 6 PER CENT. CETYL ALCOHOL AND VARYING AMOUNTS OF LIQUID PARAFFIN AND BENTONITE

| Liquid paraffin per cent. | 15 | | 20 | | 25 | | 30 | | 40 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $U_{200}$ | $f_{200}$ | $U_{200}$ | $f_{200}$ | $U_{200}$ | $f_{200}$ | $U_{200}$ | $f_{200}$ | $U_{200}$ | $f_{200}$ |
| Bentonite per cent. | Emulsions containing 0·8 per cent. sodium lauryl sulphate | | | | | | | | | |
| 0 | 2·10 | 481 | 2·65 | 541 | 2·84 | 711 | 3·68 | 894 | 5·05 | 1422 |
| 1 | 2·55 | 596 | 2·80 | 731 | 3·68 | 880 | 4·20 | 1124 | 5·23 | 2153 |
| 2 | 2·80 | 704 | 3·58 | 968 | 4·05 | 1083 | 4·72 | 1632 | 7·52 | 3142 |
| 3 | 3·35 | 907 | 3·79 | 1090 | 4·90 | 1435 | 5·85 | 2004 | 8·05 | 3941 |
| | Emulsions containing 0·4 per cent. sodium lauryl sulphate | | | | | | | | | |
| 0 | 1·92 | 372 | 2·29 | 508 | 2·84 | 697 | 3·58 | 927 | 4·72 | 1313 |
| 1 | 2·28 | 433 | 2·60 | 596 | 3·68 | 860 | 4·05 | 934 | 5·16 | 1557 |
| 2 | 2·61 | 487 | 3·28 | 738 | 3·96 | 887 | 4·64 | 1124 | 5·45 | 1652 |
| 3 | 3·09 | 623 | 3·68 | 853 | 4·57 | 1151 | 5·23 | 1415 | * | * |
| | Emulsions containing 0·2 per cent. sodium lauryl sulphate | | | | | | | | | |
| 0 | 2·32 | 345 | 2·73 | 399 | 3·09 | 528 | 3·58 | 718 | * | * |
| 1 | 2·28 | 311 | 2·65 | 345 | 3·32 | 487 | 3·91 | 609 | * | * |
| 2 | 2·51 | 359 | 3·05 | 521 | 3·54 | 542 | 4·05 | 718 | * | * |
| 3 | 2·76 | 474 | 3·54 | 596 | 3·91 | 704 | 4·72 | 927 | * | * |

* These emulsions could not be prepared by the dilution method due to instability of the primary emulsion.

*Storage.* The emulsions were kept at room temperature before the viscosity determinations which were carried out within five days of their preparation.

*Determination of the Viscosity Curve*

Two viscosity curves were drawn from measurements made at 25° C., on a rotational viscometer in which the rate of shear and torque, expressed in basic fundamental units, are calculated from the dimensions of the instrument.

1. Emulsions containing 25 per cent. liquid paraffin, from 2 to 10 per cent. cetyl alcohol, from 0·2 to 1·0 per cent. sodium lauryl sulphate, and from 0 to 3 per cent. bentonite.

2. Emulsions containing from 15 to 40 per cent. liquid paraffin, 6 per cent. cetyl alcohol, from 0·2 to 0·8 per cent. sodium lauryl sulphate, and from 0 to 3 per cent. bentonite.

## RESULTS

The results reported in this paper have been confined to those obtained from autoclaved emulsions. Autoclaved emulsions containing 25 per cent. of liquid paraffin, from 2 to 10 per cent. of cetyl alcohol, from 0·2 to 1·0 per cent. of sodium lauryl sulphate, and from 1 to 3 per cent. of bentonite, showed thixotropic uniform plastic flow, as did the autoclaved emulsions containing 6 per cent. cetyl alcohol, from 15 to 40 per cent. liquid paraffin, from 0·2 to 0·8 per cent. sodium lauryl sulphate and from 1 to 3 per cent. of bentonite. All the autoclaved emulsions containing 0 and 0·5 per cent. of bentonite showed thixotropic shear-rate thinning. The principal results of the variation of concentration of constituents on

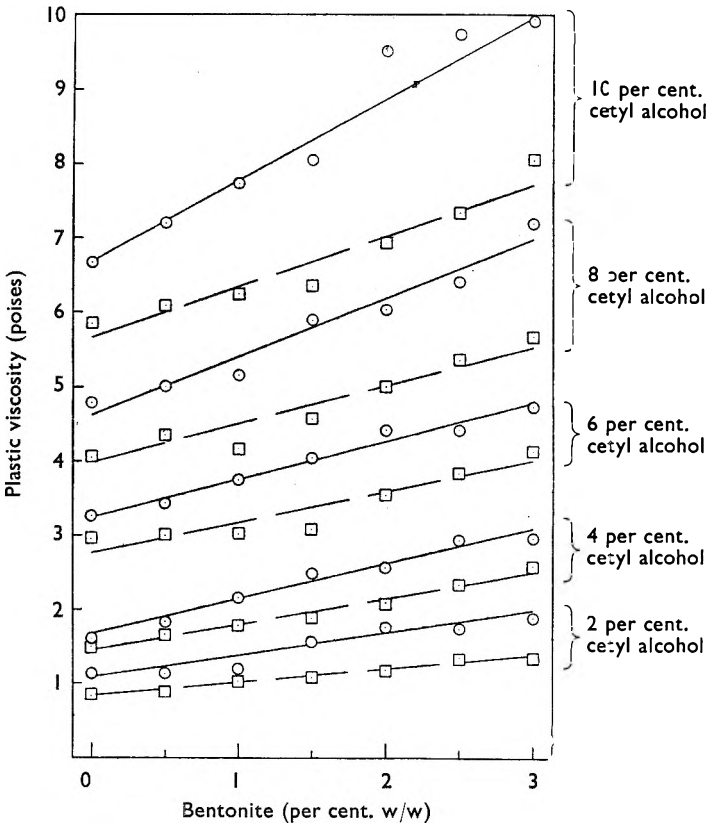


FIG. 1. The effect of the concentration of bentonite on the plastic viscosity of autoclaved oil-in-water emulsions containing 25 per cent. liquid paraffin and varying concentrations of cetyl alcohol and sodium lauryl sulphate.

⊙——⊙ 1·0 per cent. Na lauryl sulphate.
—⊡— 0·2 ,, ,, ,, ,,

emulsion consistency are summarised in Table I and in Figures 1 and 2. The quantitative values obtained for the plastic viscosity and yield value are shown in Tables II and III and Figures 1 to 6.

All the emulsions heated to 70° C., showed thixotropic shear-rate thinning. It was found that the quantitative values obtained for the limiting value of the viscosity, and the value of the torque intercept, followed an essentially similar but less distinct pattern to those shown by the autoclaved emulsions.

## DISCUSSION

The values of the plastic viscosity and yield value for autoclaved emulsions confirm the generally observed "rule of thumb".

1. It is easier to increase the consistency of an oil-in-water emulsion by increasing the amount of the oil than by increasing the primary emulsifying agent sodium lauryl sulphate. Thus in Figures 1, 2, 5 and 6

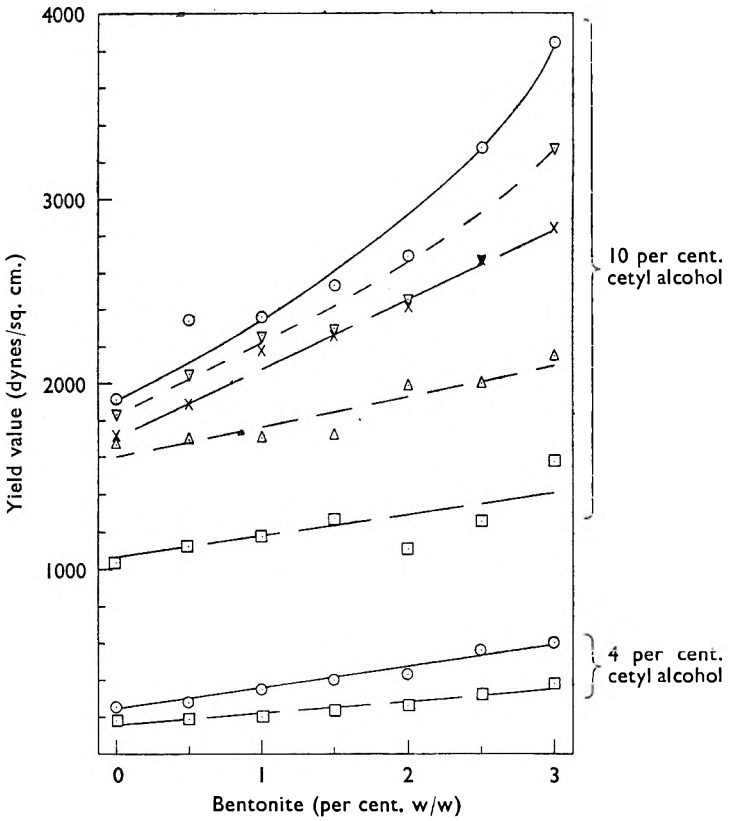


FIG. 2. The effect of the concentration of bentonite on the yield value of autoclaved oil-in-water emulsions containing 25 per cent. liquid paraffin and varying concentrations of cetyl alcohol and sodium lauryl sulphate.

⊙——⊙ 1·0 per cent. Na lauryl sulphate
–▽– — 0·8 ,, ,, ,, ,,
———×— 0·6 ,, ,, ,, ,,
— —△— 0·4 ,, ,, ,, ,,
—⊡— 0·2 ,, ,, ,, ,,

only small increases in the plastic viscosity and yield value are obtained with a several fold increase in the sodium lauryl sulphate content, while large increases are obtained for increases in cetyl alcohol or liquid paraffin.

2. A greater increase in consistency can be obtained from small increments in the cetyl alcohol content than from similar increments in the liquid paraffin content. It is seen from Figures 3 and 4 that a marked increase in viscosity is coupled with a marked increase in yield value for small increases in the cetyl alcohol content, whereas in Figures 5 and 6, similar increases in the liquid paraffin content show noticeably smaller increases in both the viscosity and yield value.

Some encouraging signs suggest that some of the qualitative terms which are in current use to describe the consistency of an emulsion can be accurately interpreted in quantitative terms. The most important general

impression which the author observed during this study was the relation of yield value to the qualitative description "body". This is particularly noticeable in the two series of emulsions containing increasing concentrations of (*a*) sodium lauryl sulphate and (*b*) bentonite, where the increase in consistency is mainly due to the increase in yield value. The yield

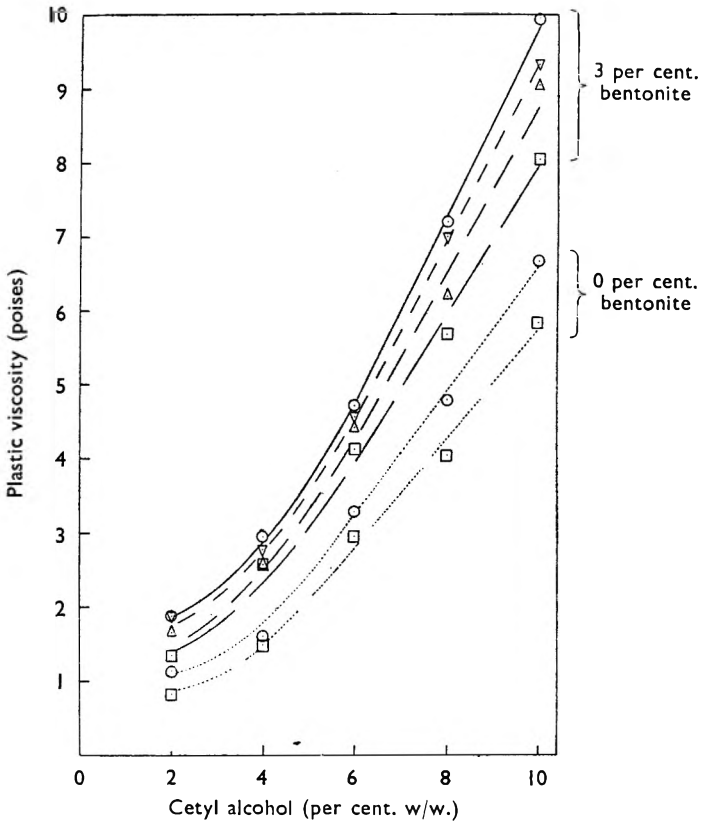


FIG. 3. The effect of the concentration of cetyl alcohol on the plastic viscosity of autoclaved oil-in-water emulsions containing 25 per cent. liquid paraffin and varying concentrations of bentonite and sodium lauryl sulphate.

| *3 per cent. bentonite* | *0 per cent. bentonite* |
|---|---|
| ⊙——⊙ 1·0 per cent. Na lauryl sulphate | ⊙ . . . . ⊙ 1·0 per cent. Na lauryl sulphate |
| ▽——▽ 0·8 ,, ,, ,, ,, | ⊡ . . . . ⊡ 0·2 ,, ,, ,, ,, |
| △——△ 0·4 ,, ,, ,, ,, | |
| ⊡——⊡ 0·2 ,, ,, ,, ,, | |

value is that force in dynes per sq. cm. which must be applied before streamline flow commences. As the yield value increases, the resistance to deformation increases, and it is this *rigidity at rest* namely yield value which appears to be comparable to the description "body" of the preparation. The qualitative expressions used may however infer differing phenomena to different investigators. Consideration of the effect of

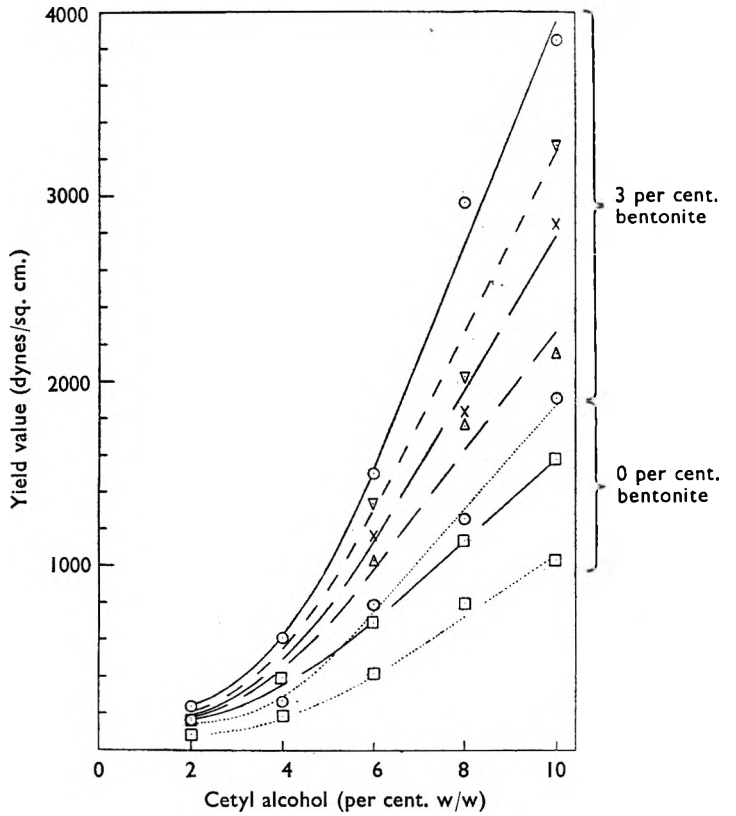


FIG. 4. The effect of the concentration of cetyl alcohol on the yield value of autoclaved oil-in-water emulsions containing 25 per cent. liquid paraffin at varying concentrations of bentonite and sodium lauryl sulphate.

*3 per cent. bentonite*

⊙——⊙ 1·0 per cent. Na lauryl sulphate
▽——▽ 0·8 „ „ „ „
×——× 0·6 „ „ „ „
△——△ 0·4 „ „ „ „
⊡——⊡ 0·2 „ „ „ „

*0 per cent. bentonite*

⊙····⊙ 1·0 per cent. Na lauryl sulphate
⊡····⊡ 0·2 „ „ „ „

other phenomena such as thixotropic breakdown with shear and with time, and the regain of the original consistency with time, will have to be investigated before the qualitative expressions are adequately characterised.

The literature review indicates that the question of globule size distribution cannot be ignored, nevertheless, this factor has not been studied in the present work. Great care has been taken to ensure a constant technique for the preparation of the emulsions studied and in view of this it has been assumed that any differences in the globule size distribution or the flocculation of the globules are a direct result of changes in the concentration of the constituents of the emulsion. When it is considered

that the autoclaved emulsions with and without bentonite were prepared from the same primary emulsion to give two different types of flow behaviour, viz., shear-rate thinning and uniform plastic flow, it is suggested that a flocculation of the globules occurs and the manner and extent of flocculation is probably more important than the globule size distribution. It is suggested as a reasonable hypothesis that plastic flow can be explained by the formation of an interlinked structure of flocculated globules within a gel network and that in emulsions which exhibit shear-rate thinning, the globules have remained dispersed or have flocculated into loose but unconnected aggregates.

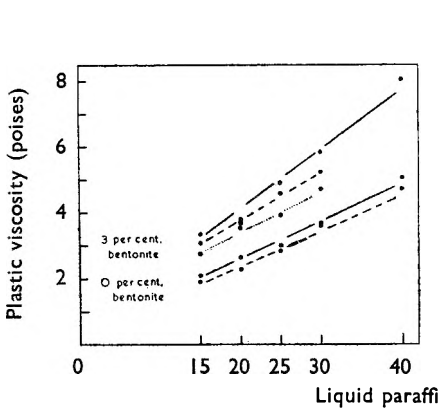


FIG. 5. The effect of the concentration of liquid paraffin on the plastic viscosity of autoclaved oil-in-water emulsions containing 6 per cent. cetyl alcohol, 0 and 3 per cent. bentonite and varying concentrations of sodium lauryl sulphate.

.... 0·2 per cent. Na lauryl sulphate
--- 0·4 " " " "
—— 0·8 " " " "

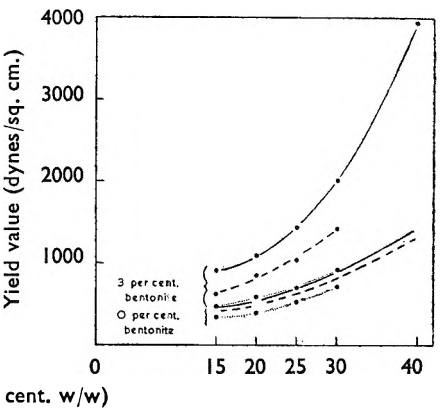


FIG. 6. The effect of the concentration of liquid paraffin on the yield value of autoclaved oil-in-water emulsions containing 6 per cent. cetyl alcohol, 0 and 3 per cent. bentonite and varying concentrations of sodium lauryl sulphate.

.... 0·2 per cent. Na lauryl sulphate
--- 0·4 " " " "
—— 0·8 " " " "

## CONCLUSIONS

1. All the unautoclaved emulsions show thixotropic shear-rate thinning.

2. Over a wide range of concentration of each constituent, the autoclaved emulsions containing from 1 to 3 per cent. of bentonite show thixotropic plastic flow.

3. The autoclaved emulsions give values for plastic viscosity and yield value which conform to a definite pattern.

4. The plastic viscosity is directly proportional to the bentonite, sodium lauryl sulphate, and liquid paraffin content of the emulsion but increases exponentially with increase in the cetyl alcohol content.

5. The yield value is directly proportional to the bentonite content up to 2 per cent., but tends to increase exponentially above 2 per cent., at the higher concentrations of sodium lauryl sulphate and cetyl alcohol. The yield value is directly proportional to the sodium lauryl sulphate content for 2, 4, and 6 per cent. cetyl alcohol and directly proportional

to the sodium lauryl sulphate content above 0·4 per cent. for emulsions containing 8 and 10 per cent. cetyl alcohol.

The yield value increases exponentially with increase in the cetyl alcohol and liquid paraffin content.


## Summary

1. A variable speed rotational viscometer was used to determine the consistency of a series of oil-in-water emulsions containing varying concentrations of liquid paraffin, sodium lauryl sulphate, cetyl alcohol, and bentonite. The results amplify the present knowledge of the effect of the concentration of constituents on the consistency of an oil-in-water emulsion system.

2. The autoclaved emulsions containing from 1 to 3 per cent. bentonite exhibited thixotropic plastic flow over a wide range of concentrations of the other constituents.

3. All the unautoclaved emulsions exhibited thixotropic shear-rate thinning.


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## DISCUSSION

The paper was presented by the AUTHOR.

MR. E. W. RICHARD (Upminster) referred to the statement on page 763 of the paper "There are conflicting reports on the effect of the globule size distribution on the consistency of oil-in-water emulsions" and said he took it that the author meant that in some cases, if the emulsions were homogenised and the globule size reduced and made more uniform, there was a thickening and in other cases a thinning. He confirmed that this happened from his own experience with different emulsions and emulsifying agents. He had found that the penetrometer, an instrument not

commonly used in pharmacy but described in the Institute of Petroleum's handbook on standard methods, was a useful empirical tool for testing semi-solid emulsions.

DR. D. TRAIN (London) suggested that there was a discontinuity in the curves shown in Figures 1 and 2 at $1\frac{1}{2}$ per cent. bentonite concentrations. Again, in Figure 5 he suggested the uppermost curve should be a shallow exponential and not a straight line.

MR. W. P. HUTCHINSON (Oxford) said that under the influence of irradiation the viscosity of certain oils changed.

MR. A. AXON, in reply, pointed out that he had quoted two authors who had stated that the effect of the emulsifying agent was most marked. He had kept to one primary emulsifying agent in the paper. He maintained that most of the effect of the emulsifying agent was to cause a difference in the aggregated state of the globules. The penetrometer was a one point instrument and had been considered previously. It gave an arbitary value which was very limited in its application for anything other than a routine control method. It would serve little purpose to attempt to distinguish between Newtonian flow, uniform plastic flow, sheer rate thinning, shear rate thickening, yield value and plastic viscosity. He had endeavoured to draw the best continuous line through the recorded points. If there is in fact a discontinuity at 1·5 per cent. bentonite concentration in Figures 1 and 2 then he was unable to give an explanation.

# NON-IONIC SURFACE-ACTIVE AGENTS

## PART I. THE SOLUBILITY OF CHLOROXYLENOL IN AQUEOUS SOLUTIONS OF POLYETHYLENE GLYCOL 1000 MONOCETYL ETHER

BY B. A. MULLEY AND A. D. METCALF

*From the Pharmaceutics Laboratories, School of Pharmacy, Chelsea Polytechnic, London, S.W.3*

Received June 26, 1956

NON-IONIC surface-active agents are being increasingly used in the pharmaceutical industry, mainly as emulsifying and solubilising agents. This paper is presented as part of a general study of the properties of these compounds and records the solubility at 20° C. of chloroxylenol (4-chloro-3 : 5-xylenol) in aqueous solutions of polyethylene glycol 1000 monocetyl ether (cetomacrogol 1000, official in the B.P.C. 1954 and B. Vet. C. 1953) in concentrations up to 20 per cent. The ultra-violet absorption spectrum of chloroxylenol in solutions of the surface-active agent is used to show that a hydrogen-bonded complex is formed between the two substances by an interaction between the phenolic hydroxyl group and the ether oxygen atoms of the polyoxyethylene glycol chain. An interpretation of the solubility curve based on the formation of the complex is given.

### EXPERIMENTAL METHOD AND RESULTS

*Materials.* Polyethylene glycol 1000 monocetyl ether of commerce (B.P.C. and B. Vet. C.) may be represented by the formula

$$CH_3{\cdot}(CH_2)_m{\cdot}O{\cdot}CH_2{\cdot}(CH_2{\cdot}O{\cdot}CH_2)_n{\cdot}CH_2OH,$$

where "*m*" may be 15 or 17 and "*n*" may be 19 to 23. The solubility of chloroxylenol in solutions of polyethylene glycol 1000 monocetyl ether was calculated using 23 and 15 as the values for "*n*" and "*m*" respectively.

Some confusion has arisen in the literature[1] concerning the correct values for "*n*". This seems to be caused by the formula given in the B. Vet. C. which we understand is to be amended. The limits for "*n*" are 19 to 23, corresponding to 20 to 24 ethylene oxide units in each molecule. It is well known[2] that commercial products of this type are not pure compounds but mixtures of materials of varying hydrophilic chain length. Statements referring to the chain lengths of these compounds normally indicate *average* chain length. The wordings of the official monographs are thus misleading.

Pure chloroxylenol was prepared by recrystallising a sample from light petroleum (b.pt. 100–120° C.) to constant m.pt. It formed colourless needles m.pt. 115·5–116° C. (uncorrected). Lesser and Gad[3] reported m.pt. 115–116° C.

Distilled water was used for all solutions.

*Method of determining the solubility of chloroxylenol in solutions of polyethylene glycol* 1000 *monocetyl ether.* A suitable range of amounts

of finely powdered chloroxylenol was weighed into a series of containers, to which were added equal volumes of a solution of the surface-active agent. The containers were immersed in a water bath at 20° C. and the mixtures equilibrated by shaking at intervals during 3 days. The limit of solubility was denoted by the appearance of turbidity in the solution, and the quantity of chloroxylenol dissolved was taken to be the mean of the amount contained in two adjacent solutions one clear the other turbid. Determinations of the solubility of chloroxylenol in solutions of the surface-active agent in concentrations of $10^{-5}$M to about 0·2M were made in a similar manner. Equilibration for longer periods did not affect the end-point, which was determined to within approximately

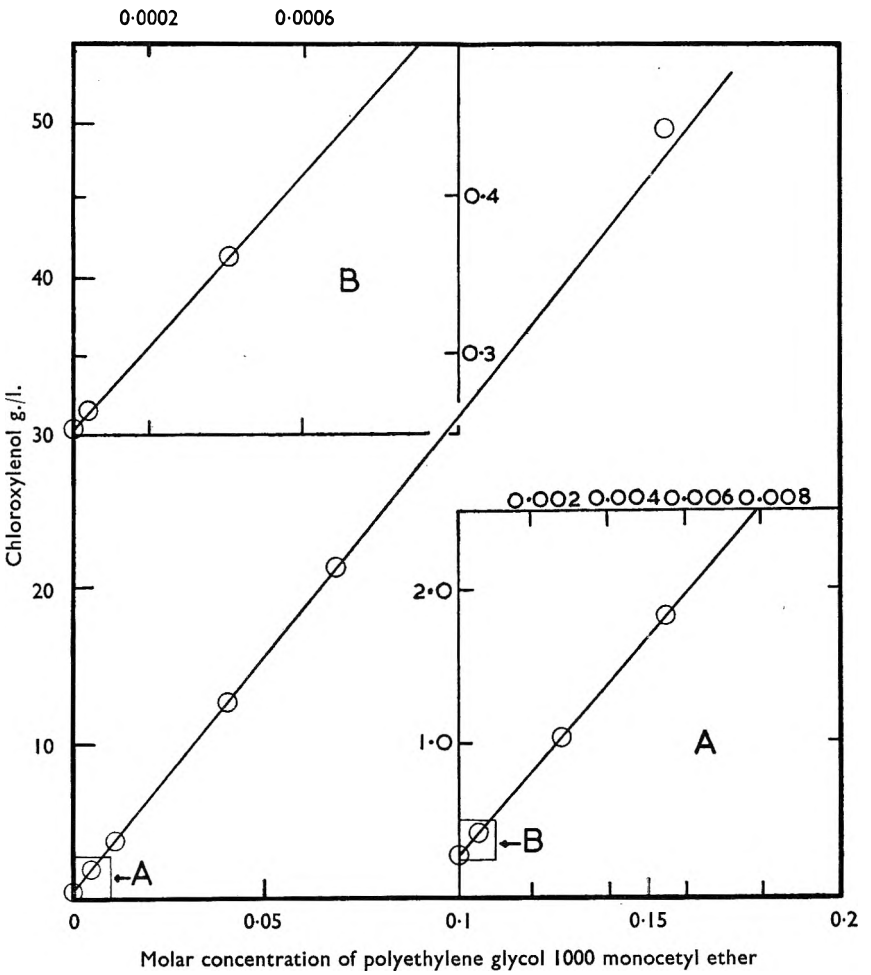



FIG. 1. The solubility of chloroxylenol in aqueous solutions of polyethylene glycol 1000 monocetyl ether. A is an enlargement of the section in the lower left corner of the diagram. B is an enlargement of the squared section of A.

±1 mg. per litre at the lower concentrations of the surface-active agent. Results are shown in Figure 1.

*The ultra-violet absorption spectra of chloroxylenol in water,* cyclo*hexane and a solution of polyethylene glycol* 1000 *monocetyl ether.* All measurements were made with a Unicam SP 500 spectrophotometer modified by Morton[4]. *cyclo*Hexane for spectroscopic purposes (B.D.H.) and distilled water were used as solvents. The spectra of chloroxylenol in water, *cyclo*hexane and 0·00109M solution of cetomacrogol are shown in Figure 2. The concentration of the phenol in the two latter solvents was approximately 1 in 2000, and in water approximately 1 in 20,000. Beer's Law was found to apply to the solution in water over the concentration range 1 in 20,000 to 1 in 40,000.

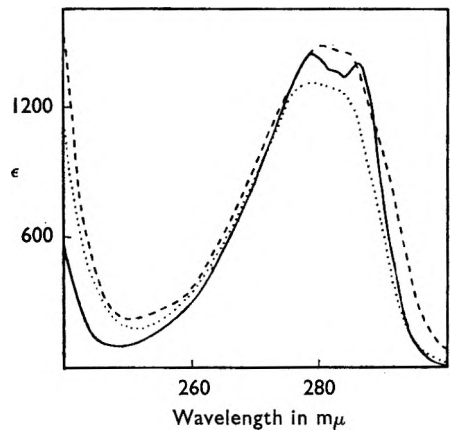



FIG. 2. Ultra-violet absorption spectra of chloroxylenol. The spectrum in *cyclo*hexane is shown by the solid line, λmax. (mμ), ε; 279·5, 1440; 286·5, 1395; inflection at 283 mμ, ε 1335. Spectrum in water (dotted line), λ max. 280 mμ, ε 1305. Spectrum in solution of cetomacrogol (broken line), λmax. 281·5 mμ, ε 1475.

*Method of determining the solubility of chloroxylenol in water.* A saturated solution of the phenol in water was prepared by shaking an excess of chloroxylenol with water at intervals for three weeks. The solution was equilibrated at 20° C. and the solubility was determined by measuring the light absorption at 280 mμ of a dilution of the saturated solution equivalent to a concentration of about 1 in 30,000 of chloroxylenol.

## DISCUSSION

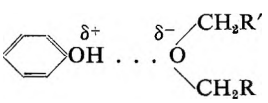
The solubility curve shown in Figure 1 is not of the form normally exhibited when water-insoluble substances are dissolved in aqueous solutions of surface-active agents. There is apparently no rapid increase in solubility at a critical micelle concentration, and the solubility of chloroxylenol, when correction has been made for the solubility in water, which has a large effect at the lower concentrations of the surface-active agent, is directly proportional to the concentration of polyethylene glycol 1000 monocetyl ether over the concentration range studied. The phenol to surface-active agent molar ratio is about 1·9 : 1. Solutions of cetomacrogol 1000 gel above 25 per cent., so the solubility of the phenol was not investigated in this region.

The critical micelle concentration of the non-ionic substances seems to be lower than that of other types of surface-active agent. Caution is necessary, however, when considering the properties of commercial materials because Kushner and Hubbard[5], using the light scattering

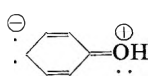
method, failed to observe a critical micelle concentration for Triton X–100, a failure which they suggested was due to the detergent being a mixture of materials of varying molecular weight. However, Gonick and McBain[6] estimated by freezing point measurements that the critical micelle concentrations of Triton X–100 and nonaethylene glycol monolaurate were $9 \times 10^{-4}$ and $6 \times 10^{-4}$ molar respectively. There are indications[7] that the critical micelle concentration may be even lower for non-ionic compounds of higher molecular weight.

Little detailed work has been published on the solubility of water insoluble substances in aqueous solutions of non-ionic surface-active agents, but it seems to be generally considered, by analogy with the soaps, that the increased solubility of such substances in solutions of the non-ionic compounds is due to the presence of micelles. The mechanism by which the presence of micelles increases the solubility has not apparently been considered in detail. McBain, Wilder and Merrill reported[8] the solubility of Orange OT in three liquid non-ionic detergents at concentrations of 0 to 100 per cent. There was no indication of a critical micelle concentration, possibly because few results were given at low concentrations of the detergents. The differential solubilisation curves showed increased solubility per mole of the detergents, particularly at concentrations greater than 20 per cent. Similar results were given in more detail for the same dye and for one of the three detergents (Triton X–100) by McBain and Green[9]. Results obtained by Hadgraft[1], working with polyethylene glycol 1000 monocetyl ether and phenols which are soluble in water, indicate that the mechanism by which phenols dissolve in solutions of non-ionic surface-active agents might be unusual. He noted that oil-in-water emulsions prepared with cetomacrogol 1000 seemed to be incompatible with phenols and suggested that this was due to the formation of a complex between them. Rough determinations made by Hadgraft of the solubilities of resorcinol and phenol in 1 to 20 per cent. solutions of cetomacrogol 1000 showed them to be less soluble than in water. This supports the suggestion that complexes are formed. A plot of Hadgraft's results shows that the solubility of these phenols is approximately proportional to the cetomacrogol 1000 concentration.

It seems most likely that the formation of complexes between phenols and non-ionic surface-active agents containing polyoxyethylene glycol chains is due to hydrogen bonding (I). It is well known that strong hydrogen bonds are formed when the hydrogen atom concerned in the bond is acidic in nature.




(I)




(II)

This probably explains the strong hydrogen bonding tendency of the phenolic hydroxy group. In terms of resonance theory the acidic nature of the phenolic hydrogen atom is due to a reduction in the electronegative nature of the oxygen atom resulting from resonance between the possible

canonical forms of the molecule, e.g. (II). Higuchi and Lack[10] showed that polyethylene glycols formed complexes with resorcinol, catechol and phenol, indicating that the interaction of phenols with non-ionic surface-active agents probably occurs at the ether portion of the molecules. The ultra-violet absorption spectra shown in Figure 2 were determined to see if hydrogen bonding between chloroxylenol and cetomacrogol 1000 could be detected. Phenols, in hydrocarbon solvents, usually show a relatively weak absorption band with well defined fine structure at about 280 m$\mu$. The results recorded in Figure 2 for chloroxylenol in *cyclo*hexane indicate that this phenol has a normal spectrum in this region. A slight shift of the band to longer wavelength, together with loss of fine structure, is observed when the solvent is water. This behaviour is typical of instances where association is possible with the solvent by hydrogen bonding[11–13]. The effect is accentuated in aqueous solutions of the non-ionic surface-active agent. It therefore seems likely that chloroxylenol is associated with the polyoxyethylene glycol chain of the surface-active agent.

When solutions of cetomacrogol 1000 are mixed with a greater amount of chloroxylenol than will dissolve, an interaction occurs and the excess material is found to be an oily liquid and not crystalline phenol unless the amount of chloroxylenol added is very much greater than the amount which will dissolve. Related instances have been reported in the literature[1,14,15]. The shape of the solubility curve in Figure 1 shows that the oily liquid is insoluble in water. The liquid probably consists of chloroxylenol together with surface-active agent and water. This has been demonstrated with *o*-chlorophenol and a non-ionic detergent[15].

The most satisfactory interpretation of the results appears to be that the solubilisation of chloroxylenol by aqueous solutions of polyethylene glycol 1000 monocetyl ether by incorporation into the micelles is probably governed by the hydrogen bonding which occurs between the phenolic hydroxyl group and the ether chain of the non-ionic surface-active agent. When the molar ratio of phenol to surface-active agent exceeds about 1·9 : 1 it seems that the hydrophilic character of the micelle is decreased and a complex separates containing surface-active agent, chloroxylenol and water. The failure to observe a rapid increase of solubility of the phenol at a critical micelle concentration may be due to the fact that the surface-active agent used was not a pure substance or that a critical micelle concentration occurs at very low concentrations at which results were not obtained owing to the experimental difficulties involved.

## Summary

1. The solubility curve of chloroxylenol in aqueous solutions of polyethylene glycol 1000 monocetyl ether at 20° C. is reported. The increased solubility of the phenol is due to its incorporation into the micelles. The curve shows that the solubility of the phenol is directly proportional to the concentration of the surface-active agent and that the solubility limit is reached at a molar ratio of phenol to surface-active agent of about 1·9 : 1.

2. Evidence is given, based on the ultra-violet absorption spectra of

chloroxylenol in *cyclo*hexane and in a solution of polyethylene glycol 1000 monocetyl ether, that hydrogen bonds are formed between the phenolic hydroxyl groups and the ether chains of the non-ionic surface-active agent.

3. It is suggested that a hydrogen-bonded complex of the phenol, surface-active agent and water is precipitated at the limit of solubility due to a reduction in the hydrophilic character of the micelles.

The authors thank Dr. H. S. Bean for help which enabled them to undertake this work. The polyethylene glycol 1000 monocetyl ether was kindly given by Glovers (Chemicals) Ltd.

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## DISCUSSION

The paper was presented by DR. B. A. MULLEY.

MR. J. W. HADGRAFT (London) wondered whether the formation of complexes accounted for the loss of activity of phenols in the presence of non-ionic surface-active agents, because possibly the affinity of the phenol for oxyethylene compounds was greater than its affinity for bacterial protein. Further, if the complex formation were concerned only with the oxygen in the ether chain of the surface-active agent, it should be possible to demonstrate the formation of complexes between phenols and the simple polyethylene glycols.

DR. L. SAUNDERS (London) said that his own work on the critical micelle concentration of non-ionic surface-active agents confirmed the conclusions of the authors. He suggested that some diffusion measurements might help in elucidating the structure of some of those complexes.

MR. J. H. OAKLEY (London) asked whether there was the same suppression of bactericidal activity if the proportion of non-ionic surface-active agent to chloroxylenol was increased over the minimum necessary for solubilisation as in the case of formulations with soap. It was well known that if more soap were put in the solution than was necessary a lowering of bactericidal effect occurred. Did the authors feel that the non-ionic approach to chloroxylenol presentation would overcome some of the incompatibilities which ionic formulations gave?

DR. B. A. MULLEY, in reply, said that the object had been to try to find out whether chloroxylenol associated with non-ionic surface-active agents. No work had been done on the activity of such solutions. There had been a suggestion in the literature that phenols and glycols formed complexes in solution, but there was a difference compared with the alkyl substituted polyethylene glycols because the complex which separated out was not solubilised by the addition of further glycol, and that made relationships completely different from the surface-active agents. He agreed that diffusion measurements might be helpful. The solubility curve method used was not sensitive enough at the very low concentrations which were probably necessary to show the critical micelle concentration. It might be that a high concentration of non-ionic surface-active agent in excess of that needed to solubilise phenol might well give lower activity. The ratio of non-ionic surface-active agent to phenol was obviously important.

# THE CHEMICAL ASSAY OF CASCARA

By A. A. J. Fluck, Wm. Mitchell and S. A. Wood

*From Stafford Allen & Sons, Ltd.*

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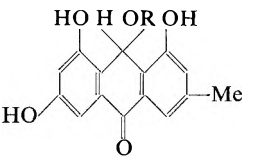
The bark of cascara (*Rhamnus purshianus* DC.) is believed to contain free emodin, aloe-emodin, and chrysophanol. The purgative activity of the drug is thought to be due not to these free anthraquinones, but to glycosides of the anthranols (or of the oxanthrones) corresponding to some or all of them. Little is known about the structure of such glycosides, except that Schindler[1] has reported the isolation of a glycoside of emodin oxanthrone (I).




(I) R = glucose

Several methods for the assay of cascara have been published. Some of these attempt the determination of total anthraquinones, usually by colorimetric measurement of the red colour produced by the action of alkali on anthraquinones (Borntträger reaction). The results so obtained cannot be expected to measure the therapeutic activity of the drug if, in fact, this activity resides mainly or entirely in the glycoside derivatives.

Kussmaul and Becker[2] described a method for the assay of the sennosides (the glycosides isolated from senna by Stoll, Kussmaul and Becker[3]). They also applied the method to the assay of glycosides in the crude drug, and in galenicals prepared from it. Fairbairn[4] and others have developed this method, and applied it to senna[5–8], rhubarb[9], and cascara[10]. Fairbairn and others also used, for comparison, a biological assay devised by Lou[11].

There is certainly a need for a reliable assay of cascara preparations. The British Pharmacopœia standards for the dry extract (the preparation probably used in greatest amount) are of little value in determining its quality and therapeutic value. In fact, there are extracts on the market that answer the B.P. requirements, yet are sold at a price that would scarcely cover the cost of the cascara bark required to make a B.P. extract. Such products are evidently suspect. One such extract was kindly tested for us by Dr. Fairbairn, using Lou's method. It was found to have an extremely low purgative action, while results obtained by Fairbairn and Mahran's[10] chemical assay method were also unusually low. It is to be expected that the cheapest cascara extracts that meet the requirements of the British Pharmacopœia will often be used. If such extracts, and in particular, tablets made from them, are of low therapeutic value, it is

obvious that cascara may become unjustifiably discredited. It was these considerations that prompted us to commence the work, the first part of which is described in the present paper.

## Experimental and Results

*Fairbairn and Mahran's Method*[10]

Initially, we applied this method to dry extract of cascara (the same sample was used throughout the work now described). This method comprises the following steps.

1. Chloroformic extraction of free anthraquinones from an aqueous solution of the extract adjusted to pH 3 with hydrochloric acid.
2. Hydrolysis of the glycosides by heating the extracted aqueous solution, adjusted to about 3·3N with sulphuric acid after the addition of hydrogen peroxide, for 15 minutes in a boiling water bath.
3. Ether extraction of liberated aglycones from the acid mixture. The ether solution is successively washed with several portions each of 10 per cent. w/v aqueous sodium metabisulphite and of 1 per cent. w/v aqueous sodium bicarbonate.
4. Extraction of the aglycones from the washed ether solution by shaking with N sodium hydroxide, followed by oxidation with hydrogen peroxide to convert any reduced forms into anthraquinones. The solution is acidified, and extracted with ether. The ether is re-extracted with N sodium hydroxide. From the absorption of the alkaline extract at 500 mμ (photo-electric measurement) the content of aglycones, calculated as aloe-emodin, is determined from calibration curves prepared from tests on pure aloe-emodin. The results are calculated as percentages of aloe-emodin in the sample.

Our results by this method were far from satisfactory, ranging from 0·6 to 1·4 per cent. It was soon realised that variations were mainly determined by the hydrolysis procedure; that the prescribed conditions did not appear to secure complete hydrolysis; and that more prolonged heating sometimes gave higher results. Furthermore, solutions from which the aglycones had been completely extracted were found, after re-heating, *especially* if additional hydrogen peroxide were added, to yield a further quantity of material behaving as aglycone; and still further quantities could be obtained by repetition of this treatment, and without reaching finality. We also found that the colour of the final test solutions faded rapidly on exposure to daylight. Besides avoiding such exposure, we adopted the practice of taking spectrophotometric readings within 30 minutes, whenever possible. Table I gives the ranges of results obtained under various conditions of hydrolysis. The variations were large and unpredictable, and we were unable to find any procedure that gave consistent results.

Hydrogen peroxide was added to the hydrolysis mixture by Fairbairn and Mahran to aid in obtaining the final test solution free from interfering absorption. This was also the purpose of the washing of the ethereal solution with sodium metabisulphite and with sodium bicarbonate. The

TABLE I

COMPARATIVE ASSAY RESULTS OBTAINED ON A SAMPLE OF DRY EXTRACT OF CASCARA, B.P. BY THE FAIRBAIRN AND MAHRAN METHOD[10], AND MODIFICATIONS OF IT

| Hydrolysis procedure | | Aloe-emodin* (per cent.) |
|---|---|---|
| (*a*) | as described by Fairbairn and Mahran: 15 minutes in boiling water bath | 0·6 to 1·4 |
| (*b*) | 1 hour in boiling water bath | 1·0 to 1·6 |
| (*c*) | 2 hours in boiling water bath | 1·3 to 1·6 |
| (*d*) | boiled under reflux for 15 minutes | 0·7 to 1·1 |
| (*e*) | boiled under reflux for 1 hour | 0·2 to 0·5 |
| (*f*) | 3 periods each of 15 minutes in boiling water bath, with addition of fresh peroxide each time. Aglycones isolated after each treatment. | 1st 1·14<br>2nd 0·10<br>3rd 0·16 |
| | Total | 1·40 |

* Our specimen of pure aloe-emodin, in N sodium hydroxide, had $E$ (1 per cent. 1 cm.) = 345 at 500 mμ; this figure was used to calculate these and all subsequent results quoted.

combined effect of these treatments was claimed to eliminate interfering matter, so that the shape of the absorption curve was similar to that of aloe-emodin in alkaline solution. In our hands this was not so. In nearly all instances there was considerable absorption around 400 mμ (Fig. 1), though occasionally, and unaccountably, the form of the curve was much nearer to that of aloe-emodin. If one assumes that the extraneous absorption is caused by material unrelated to the active principles, and this remains to be proved, it can be expected that its presence also causes absorption at 500 mμ, and hence an elevation of the apparent

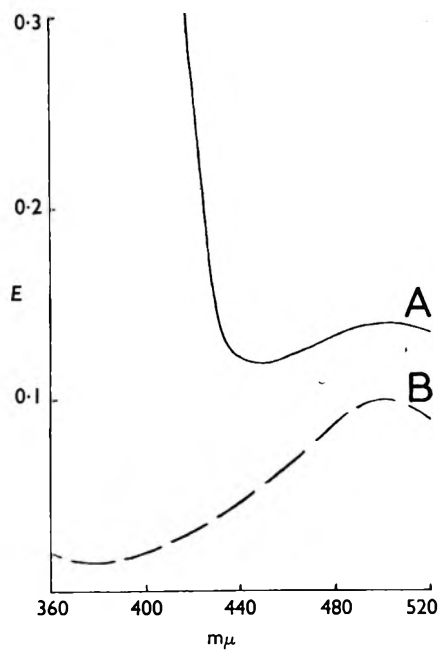



FIG. 1. Absorption curves of (A) cascara aglycones separated by Fairbairn and Mahran's process[10], and (B) aloe-emodin (both in N sodium hydroxide solution).

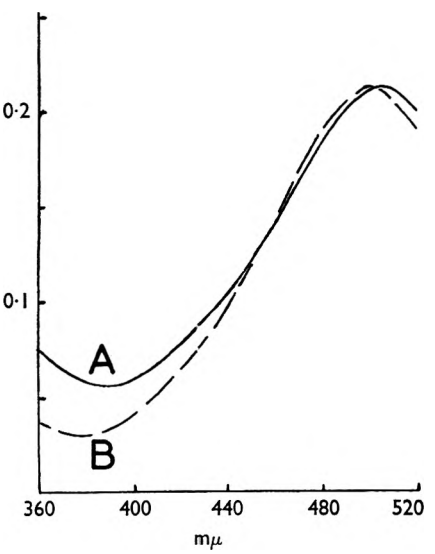



FIG. 2. Absorption curves of (A) light petroleum-extracted cascara aglycones, and (B) aloe-emodin (both in N sodium hydroxide solution).

figures for aloe-emodin. This may account for some of the variation in the above results.

Control experiments with comparable quantities of aloe-emodin, under the prescribed hydrolysis conditions, showed losses of about 20 per cent.; the same applied to alizarin, or to the aglycones isolated from cascara. More prolonged heating increased the loss, which became complete when the mixture was boiled for 30 minutes. Curiously, there is a small loss of aloe-emodin with sulphuric acid alone; but no loss when an equivalent amount of hydrochloric acid (without peroxide) is used instead. These findings caused us to use hydrochloric acid only in our later work.

Because of this action of hydrogen peroxide, it is surprising that any aglycones survived the more prolonged hydrolyses, and we can only speculate about the reason. It is possible that the peroxide is rapidly consumed in attacking other, more readily oxidisable, material. On the other hand, we have found, as mentioned above, that re-heating with fresh peroxide seems to produce more anthraquinone bodies. Whether or not these are derived from therapeutically useful substances remains to be determined. In any event, the results obtained by hydrolysis after the addition of peroxide may well be resultants of simultaneous liberation and partial destruction of anthraquinone derivatives, so that considerable variations could be expected. Accordingly we abandoned the use of peroxide as unreliable.

Similarly, we cannot recommend the use of peroxide in the final (alkaline) stage. Control experiments with aloe-emodin showed a loss of about 10 per cent. under the prescribed conditions, and this was confirmed with algycone solutions prepared from cascara. Furthermore, it appears to us that oxidation is already complete without the addition of peroxide.

*Acid Hydrolysis, Benzene Extraction and Chromatography*

We then conducted a series of experiments involving hydrolysis of the extract by boiling under reflux for 30 minutes with 3·3N hydrochloric acid, followed by extraction with solvents other than ether. Of those tried, light petroleum (b.pt. 40° to 60° C.) proved highly selective. Direct extraction of it with N sodium hydroxide gave rose-red solutions having spectral curves closely similar to that of pure aloe-emodin (Fig. 2). Unfortunately, the aglycones are so sparingly soluble in light petroleum that it was difficult to secure complete extraction.

Extraction with benzene proved more practicable, but it was considerably less selective than light petroleum, though more so than ether. Partition chromatography on silica gel containing 15 per cent. of water effected considerable improvement, the impurities remaining as a brown band on the upper part of the column.

Further determinations were accordingly made by the following procedure.

Dry cascara extract B.P. (about 40 mg., accurately weighed) was dissolved in water (20 ml.), hydrochloric acid was added to pH 3, and free anthraquinone derivatives were extracted with benzene (5 portions, each of 50 ml.). Each benzene extract was washed with the same portion

(10 ml.) of water. The united benzene solutions were extracted with N sodium hydroxide (4 portions, each of 10 ml.). Residual benzene was removed by subjecting the cold alkaline solution to high vacuum. After suitable dilution, it was examined spectrophotometrically.

The acid aqueous liquid, so extracted, and the water washing were mixed with concentrated hydrochloric acid (15 ml.), and the mixture was boiled under reflux for 30 minutes. The cooled mixture was extracted with benzene (4 portions, each of 25 ml.). The total benzene solution was passed through a 6 cm. × 22 mm. diameter column of silica gel (80/170 mesh) containing 15 per cent. of water. The column was eluted with benzene containing 10 per cent. v/v of chloroform, a bright yellow band gradually passing downwards. Elution was continued until the band was completely removed. The united eluate was extracted with N sodium hydroxide (4 portions, each of 5 ml.). The alkaline solution, after freeing from benzene *in vacuo*, was examined spectrophotometrically in suitable dilution.

The results, and the shape of the curves, for the free anthraquinones were quite constant. The curves showed a peak at 510 mμ, and some interfering absorption (Fig. 3). The relative freedom from interfering absorption suggests that the material causing it is mainly liberated on acid hydrolysis. The results ranged from 0·58 to 0·67 per cent., calculated as aloe-emodin (from the absorption at 510 mμ and using the value $E$(1 per cent. 1 cm.) = 345).

The results for the aglycones liberated by acid hydrolysis were much less satisfactory. The form of the curves obtained was reasonably good, but with the peak at 505 mμ. (Fig. 3). The results were variable, ranging from 0·3 to 0·6 per cent., calculated as aloe-emodin (from the absorption at 505 mμ, and using the value $E$(1 per cent. 1 cm.) = 345). More prolonged hydrolysis sometimes gave slightly lower results.

These results were distinctly lower than those we obtained by the Fairbairn and Mahran method. This could be due to elimination of interfering matter that had raised the former results. Again, losses at the chromatographic stage are possible, though control experiments with pure aloe-emodin had given virtually complete recovery. It is also possible that the cascara aglycones are destroyed by boiling with hydrochloric acid, even though aloe-emodin is not. However, the results were still so variable that it was difficult to draw any valid conclusion. Further experiments in which hydrazine hydrochloride was added to the acid hydrolysis mixture, in an attempt to avoid possible oxidative destruction, gave equally unsatisfactory results. Accordingly, we decided that methods involving hot acid hydrolysis were unlikely to yield results of value.

Hydrolysis by boiling for 30 minutes with 10 per cent. w/v aqueous sodium hydroxide was also tried. Much coloured material, but very little aglycone was produced; and subsequent reboiling of the extracted residue, after adjustment to 3·3N with hydrochloric acid, produced only a negligible further amount of aglycone. Thus it appears that alkaline hydrolysis destroys most of the aglycones.

At about this time we obtained an interesting result. The free anthraquinone derivatives had been removed, by chloroform extraction, from a solution of dry extract of cascara, B.P. (1 g.) in water (500 ml.), after adjustment with hydrochloric acid to pH 3. This had been done for convenience, so that aliquot portions could be used directly for hydrolyses under various conditions. The initial results were in good agreement at about 0·62 per cent. of aloe-emodin. Further tests were delayed for 13 days, when the results, again in good agreement, were about 0·78 per cent. This increase of some 25 per cent. surprised us. The solution was

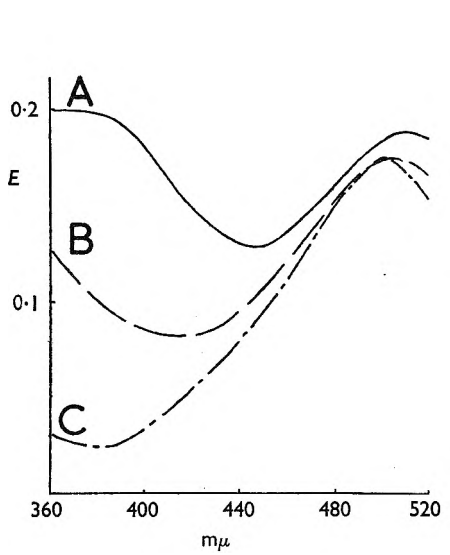



FIG. 3. Absorption curves of (A) free anthraquinone compounds, (B) aglycones (both separated from cascara by the method described in this paper), and (C) aloe-emodin (all in N sodium hydroxide solution).

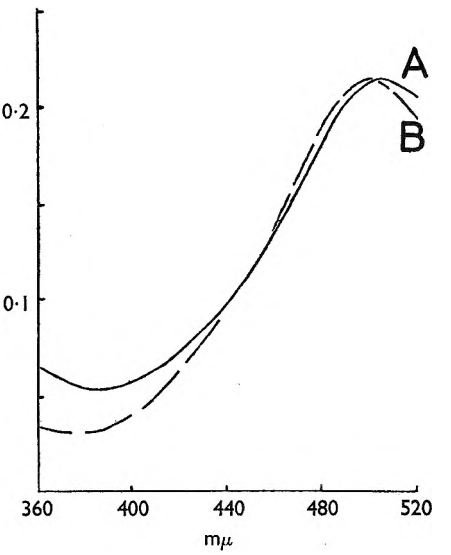



FIG. 4. Absorption curves of (A) cascara aglycones produced by slow hydrolysis in the cold, and (B) aloe-emodin (both in N sodium hydroxide solution).

tested for the presence of aglycones by benzene extraction, and direct extracton of the benzene (not chromatographed) with N sodium hydroxide. A rose-red solution was obtained, despite the initial complete removal of free anthraquinone derivatives from the cascara solution. This indicated that slow hydrolysis had occurred on standing. More important, the spectral curve of the aglycones (Fig. 4) was almost identical with that of pure aloe-emodin, and the absorption at 505 mμ corresponded to 0·37 per cent. of aloe-emodin. After a total of 8 weeks, a further test showed 0·82 per cent. of free aglycones as aloe-emodin, still with no interfering absorption. This result, higher than that initially obtained by hot acid hydrolysis, suggests that such treatment may destroy some of the aglycones. It certainly shows that slow hydrolysis in the cold liberates aglycones without simultaneous production of interfering matter (*cf.* the relative freedom from extraneous absorption of the solution of free anthraquinone derivatives isolated from the extract).

*Hydrolysis Under Mild Conditions*

Attempts were then made to effect hydrolysis under similar mild conditions, but in a reasonably short time. It was found that the aglycones could be completely extracted by chloroform without pH adjustment. A solution, so freed, was boiled under reflux at the natural pH (about 5·6) for 4 hours. Little or no hydrolysis occurred. This result would imply that hydrolysis of glycosides should not occur during the ordinary processes of manufacture of cascara extracts; though loss of potency may be caused (as suggested by Fairbairn and Mahran's results[10]), due to other changes of unknown nature. In further experiments, adjustment with hydrochloric acid to pH 3·0 preceded refluxing. Even after 4 hours, results of only 0·31 per cent. of aloe-emodin were obtained, while interfering absorption was observed. Standing for 3 days at room temperature in the presence of 3·3N hydrochloric acid gave only 0·37 per cent. of aloe-emodin, and interfering absorption was again observed.

We are forced to conclude that the method of Fairbairn and Mahran cannot be expected to give satisfactory results with cascara. This is disappointing, in view of the need for a reliable assay. Although our results so far have been almost entirely negative, we consider that it may be helpful to other workers to record them. The work described has suggested to us other lines of approach that are now being investigated.

## Summary

1. The chemical assay of dry extract of cascara B.P. has been studied.

2. The method of Fairbairn and Mahran[10] has been found to be unreliable. In particular, the addition of hydrogen peroxide in hydrolysis causes partial destruction of anthraquinones, yet it also appears to liberate additional anthraquinone-like material *ad infinitum*.

3. Light petroleum effects selective extraction of the aglycones, but the process is difficult to complete. A method involving acid hydrolysis, benzene extraction, and partition chromatography is described. Though the aglycones were thus obtained in relative purity, the quantitative results were disappointing, and the method cannot be recommended as an assay process.

4. Prolonged hydrolysis in the cold gives aglycones free from interfering matter, but could not be used in a practicable assay method.

We wish to thank Miss H. M. Perry for assistance with some of the analytical work; and Dr. J. W. Fairbairn for the biological test of a commercial sample of dry extract of cascara B.P.

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## DISCUSSION

The paper was presented by DR. W. MITCHELL.

The CHAIRMAN expressed the hope that the authors would attempt to ascertain what happened during a standing period of 26 days.

DR. F. HARTLEY (London) said that the standardisation of cascara was a great challenge. DR. Mitchell had shown that the Fairbairn and Mahran method was unreliable but he had not put forward an alternative method. Many people had reached the same position as the authors, but they had not yet thought it appropriate to publish that work. DR. MITCHELL had obtained higher figures by long term hydrolysis but he had not checked to see what he was determining. It was not known whether the physiological potency was due to glycosides or aglycones.

DR. J. W. FAIRBAIRN (London) said that as a result of Dr. Mitchell's criticisms he had tried two other examples using his own method and obtained reasonably consistent results, e.g., 9·63 mg./g. and another worker 9·0 mg./g., for one sample, but he was sorry to have to confess that when he continued the work he found two rather serious defects, especially with B.P. extracts. First of all, the final colour was not always as pure as it should have been. Secondly, the method did not go to completion as one could continue to obtain more aglycones from the extract after hydrolysis was finished. That had led him to investigate the problems carefully, and he had arrived at the conclusion that there were apparently three glycosides present in cascara; based on (1) emodin (2) chrysophanol and (3) aloe-emodin. The emodin glycoside was easily hydrolysed, in fact, very little survived in the extract, and this possibly explained why curve A in Figure 3, had a peak at about 510 mμ. The peak for emodin is 520 mμ whereas for aloe-emodin and chrysophanol it is 500 mμ thus indicating that in the B.P. extract, emodin occurred as aglycone and not as a glycoside The second glycoside based on chrysophanol seemed less easy to split and survived as a glycoside in the extract, while the third glycoside, aloe-emodin, was difficult to hydrolyse and was possibly the same glycoside as was isolated by Lee and Berger. He had a feeling that the interfering substance was attached to that glycoside. By careful preliminary treatment he had been able to induce the separation of this interfering substance and produce a large measure of hydrolysis. Thus with one sample of extract he had obtained 11 mg./g. of impure glycoside by the old method, but by the new method he obtained 29 mg./g. of very pure material. The new method had been tried on several extracts and it appeared as though the order of activity, as compared with the biological activity, was the same as with the old method. By the new method he still found that the bark was much more active than the extract in proportion to the amount of glycoside, and it was tempting to suggest that the

easily split glycoside which disappeared was responsible for the higher activity. He had not found any loss in pure aloe-emodin treated by his method of oxidation in caustic soda, nor did he find the colour faded on standing, except possibly, in sunlight.

DR. D. C. GARRATT (Nottingham) said he had come to the conclusion that the activities and the results of chemical assay of commercial products were so diverse that it was necessary to adopt another line of approach. The difficulty was that if the extract were kept for any length of time, the biological and chemical assays did not agree. It was difficult to see why a purified active principle or a material such as senna powder itself, which was reasonably uniform in activity, could not be used as a standard.

DR. J. W. ROWSON (London) said that the details of the "interesting result" on page 786 of the paper were not clear. Were the "results in good agreement" obtained by the previously described hydrolysis method which had been already rejected as non-reproducible? If the figure of 0·78 per cent. included 0·37 per cent. of free aglycones, was the 0·82 per cent. free aglycones after 8 weeks also the total or were still further quantities combined?

MR. T. D. WHITTET (London) said that he had prepared extracts by four methods, and Dr. Fairbairn had arranged for some preliminary assays. There was some evidence that there was a considerable loss of activity in all.

DR. W. MITCHELL, in reply, said that for simplicity the results reported in the paper were obtained with one particular extract. Many other extracts had been examined, but it would have led to complication to set out a number of results. The title of the paper was "The Chemical Assay of Cascara" and the first object was to try to find an assay which would give reproducible results, the next stage would be to try to relate them with biological findings. At the present stage all that could be indicated was that the methods available were not capable of giving good results. However, further work, which it was hoped to publish in due course, had led to a method which, with the same extract, gave results as high as 60 mg./g., with no trouble due to interfering absorption, and with good replication of results. Furthermore, the chemical figures on the extract were in quite good correlation with those of the sample of bark from which the extract was made. Replying to Dr. Rowson he said that after standing in the cold at pH 3, anthraquinones appeared in material previously freed from aglycones. These anthraquinones could be extracted and submitted to spectrophotometric examination when no interference was detected. Arising from Dr. Fairbairn's remarks, he commented that his own recent work had caused him to doubt whether the active principles were true glycosides.

# A SOURCE OF ERROR IN THE ASSAY OF STRYCHNINE SALTS AND PREPARATIONS CONTAINING STRYCHNINE

By A. C. Caws and G. E. Foster

*From the Wellcome Chemical Works, Dartford*

Received June 21, 1956

Strychnine, the most important alkaloid obtained from *Strychnos nux vomica* L., was discovered in 1817 by Pelletier and Caventou[1] and since that time has been widely used in medicine. On this account the assay of nux vomica and of strychnine preparations has been investigated many times and such assays are now carried out by a few well established procedures. Owing to the sparing solubility of the alkaloid in most common solvents chloroform, of which six parts dissolve one of strychnine, has been almost universally used as solvent for extraction of the alkaloid from preparations being assayed for strychnine. It has been usual for the solvent to be removed from the chloroform extract by distillation, after which the residual base may be either weighed, after drying to constant weight, or titrated with standard acid. Two colorimetric methods for completing the assay have found considerable application, particularly when small amounts of strychnine are to be estimated. Denigès[2] modification of Malaquin's colour test has been critically studied by Allport and his collaborators[3,4] with the object of using it for colorimetric determination of strychnine. In this procedure the alkaloid is reduced in acid medium with zinc amalgam and the product treated with sodium nitrite solution when the red colour produced is a measure of the strychnine present. The colour developed with ammonium vanadate in the presence of sulphuric acid[5] also affords a satisfactory means of colorimetric assay. The extent to which these procedures have found favour is indicated in Table I, which gives a summary of the methods employed by some official and semi-official books of standards.

In our laboratories, where strychnine salts and preparations are examined, the analytical methods used cover all of those included in Table I. We have repeatedly found, however, that there was a small discrepancy between the results obtained when the residue from the chloroform extract was weighed as strychnine and when it was titrated with standard acid, the former always giving the higher result. A series of assays carried out by independent workers convinced us that the observed difference was not due to experimental error and it was therefore decided to examine critically the assay procedures. The present paper describes the results of this investigation.

## Experimental and Results

### *The Assay of Strychnine Salts*

The B.P. method for the assay of strychnine hydrochloride consists of extraction with chloroform of the alkaloid from a solution of the salt, rendered alkaline by addition of dilute solution of ammonia, evaporation

TABLE I

SUMMARY OF OFFICIAL AND SEMI-OFFICIAL ASSAY PROCEDURES

| Publication | Preparation | Solvent used | Method for estimating strychnine in residue from extract |
|---|---|---|---|
| B.P. 1953 | Nux vomica, its dry extract, liquid extract and tincture | Chloroform | Titration with standard acid |
| B.P. 1953 | Strychnine hydrochloride and solution of strychnine hydrochloride | Chloroform | Titration with standard acid |
| B.P.C. 1954 | Strychnine sulphate | Chloroform | Titration with standard acid |
| B.P.C. 1954 | Injection of strychnine and mixture of strychnine | Chloroform | Colorimetric estimation using solution of ammonium vanadate as reagent |
| B.P.C. 1954 | Easton's syrup and tablets of Easton's syrup | Chloroform | Weigh residue after drying to constant weight at 105° C. |
| National Formulary IX | Strychnine phosphate, tablets of strychnine nitrate and tablets of strychnine sulphate | Chloroform | Titration with standard acid |

of the solvent from the extract and titration of the residue with standard acid using solution of methyl red as indicator. We have used this official procedure for the assay of strychnine salts but the analytical figures obtained have always been lower than those recorded when the final residues were weighed as strychnine instead of being titrated. In some experiments the residues were titrated potentiometrically but the results were identical with those obtained using methyl red as indicator. Some typical results are shown in Table II.

TABLE II

COMPARISON OF RESULTS BY WEIGHING AND TITRATION

| Salt | Sample | $C_{21}H_{22}O_2N_2$ content, expressed as per cent. in undried sample | |
|---|---|---|---|
| | | From weight of base | By titration of base |
| Strychnine sulphate .. .. | 1 | 78·8 | 76·6 |
| " " .. .. | 2 | 78·3 | 76·4 |
| " " .. .. | 3 | 79·6 | 76·2 |
| Strychnine hydrochloride .. | 1 | 84·1 | 80·8 |
| " " .. | 2 | 84·0 | 81·4 |
| " " .. | 3 | 84·6 | 80·9 |

*Chemical Reaction between Chloroform and Strychnine*

Our work on strychnine salts suggested that some reaction between the alkaloid and chloroform may occur during the assay and in order to investigate the possibility weighed portions of pure strychnine base were transferred to tared flasks, 100 ml. portions of chloroform added to each and the resulting solutions evaporated to dryness. The residues were weighed after drying at 105° C, and then titrated with standard acid. Particular attention was paid to the removal of the chloroform from the solutions and, in selected experiments, the following three procedures were adopted: (1) Evaporation without the addition of ethanol. (2) Evaporation to small volume, addition of 5 ml. of ethanol

and evaporation to dryness (B.P. method). (3) Evaporation to about 20 ml., evaporation continued to dryness while two portions (10 ml.) and three portions (5 ml.) of ethanol were added.

Table III summarises the results of these experiments in which strychnine, containing 98·9 per cent. $C_{21}H_{22}O_2N_2$ estimated by titration, was used.

TABLE III

ESTIMATION OF STRYCHNINE USING THREE PROCEDURES OF SOLVENT REMOVAL

| Weight of strychnine | Procedure for removing solvent | Weight of base recovered | Percentage of strychnine recovered | |
|---|---|---|---|---|
| | | | By weight | By titration |
| 0·5039 g. | 1 | 0·5123 g. | 101·7 | 96·1 |
| 0·5018 g. | 2 | 0·5085 g. | 101·3 | 97·7 |
| 0·4895 g. | 3 | 0·4935 g. | 100·8 | 96·5 |

The final titration liquors from these experiments were acidified with nitric acid and tested for ionisable chlorine by addition of silver nitrate solution; in all cases chloride was found to be present. In order to investigate the formation of ionisable chlorine quantitatively a further series of experiments was performed in which the final titration liquors, obtained during the strychnine determination, were titrated electrometrically with 0·02 N silver nitrate solution. All three methods of solvent evaporation were employed and, in some cases, chloride formation was increased by boiling the alkaloidal solution under a reflux condenser for periods up to 5 hours before the chloroform was removed by evaporation. In extension of this work, the amount of alkaloid used was increased to allow the base recovered from the chloroform solution to be divided into two portions, one of which was titrated in the usual manner and the other being dissolved in glacial acetic acid and titrated with 0·1 N perchloric acid solution after the addition of mercuric acetate, solution of crystal violet in glacial acetic acid being used as an indicator. A summary of the results obtained is given in Table IV.

It was at first concluded that our experimental results could be explained by the formation of a small amount of strychnine hydrochloride, due to the interaction of the alkaloid and chloroform, but this conclusion proved to be erroneous as the increase in the weight of the base on evaporation of its solution was far greater than that due to the ionisable chlorine found.

*Isolation of Chloro-compound*

During experiments in which solutions of strychnine in chloroform were boiled for some hours it was noticed that an insoluble product gradually separated from the solution and first appeared after one or two hours boiling. The yield of material could be increased by continuing the boiling for several days and in this way sufficient was collected for examination. By recrystallisation from water, it was obtained as colourless needles, melting with decomposition above 300° C. The following analytical figures were obtained using a sample dried at 105° C. Found: C, 57·2; H, 5·3; N, 6·63; Cl, 17·5; ionisable Cl 7·65 per cent.

TABLE IV

SUMMARY OF ANALYTICAL RESULTS

| Expt. No. | A Assay by titration on original strychnine | Weight of strychnine taken g. | Method of evaporation | Weight of strychnine recovered g. | Strychnine recovered by weight per cent. | B Strychnine recovered by 0·1 N $H_2SO_4$ titration per cent. | Ml. 0·02 N $AgNO_3$ required for $Cl'$ | Difference per cent. between A and B | | Strychnine recovered by 0·1 N $HClO_4$ titration per cent. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Calculated from $AgNO_3$ titration | Found | |
| 1 | 98·3 | 0·5180 | 1 | 0·5309 | 102·5 | 96·25 | 1·90 | 2·5 | 2·05 | — |
| 2 | 98·5 | 0·4464 | 1 | 0·4566 | 102·3 | 97·4 | 0·97 | 1·45 | 1·1 | — |
| 3 | 99·2 | 1·3044 | 1 | 1·3230 | 101·5 | 97·6 | 3·77 | 1·8 | 1·6 | 99·8 |
| 4 | 98·3 | 0·5496 | 2 | 0·5569 | 101·3 | 96·2 | 2·15 | 2·6 | 2·1 | — |
| 5 | 98·5 | 0·4490 | 2 | 0·4555 | 101·5 | 97·5 | 1·05 | 1·6 | 1·0 | — |
| 6 | 99·2 | 1·2348 | 2 | 1·2557 | 101·8 | 98·0 | 3·38 | 1·7 | 1·2 | 99·7 |
| 7 | 98·3 | 0·5741 | 3 | 0·5768 | 100·5 | 96·2 | 1·90 | 2·25 | 2·1 | — |
| 8 | 98·5 | 0·4579 | 3 | 0·4620 | 100·9 | 97·5 | 1·00 | 1·5 | 1·0 | — |
| 9 | 99·2 | 1·2180 | 3 | 1·2295 | 100·9 | 98·3 | 2·49 | 1·2 | 0·9 | 100·0 |
| 10* | 98·5 | 0·4474 | 1 | 0·4616 | 103·2 | 95·3 | 2·65 | 4·0 | 3·2 | — |
| 11* | 99·2 | 1·3020 | 1 | 1·3329 | 102·4 | 94·4 | 10·00 | 5·0 | 4·8 | 99·9 |
| 12* | 99·2 | 1·0347 | 1 | 1·0677 | 103·2 | 92·8 | 10·3 | 6·5 | 6·4 | 99·6 |
| 13* | 99·2 | 1·0095 | 1 | 1·0599 | 105·1 | 90·2 | 14·34 | 8·7 | 9·0 | 100·0 |

* In Expts. 10 and 11—strychnine boiled under reflux with chloroform for 2–3 hours; in Expt. 12 for 3 hours and in Expt. 13 for 5 hours; before evaporation by method 1.

Equivalent weight by (*a*) titration with 0·1 N silver nitrate = 463·4, (*b*) non-aqueous titration with 0·1 N perchloric acid = 464·4. $[\alpha]_D^{20} + 17°$ (*c.*, 2 in water). Examination by paper chromatography indicated that

the material consisted of a single substance. For this purpose 0·01 ml. of a 1 per cent. w/v solution in ethanol was placed on a strip of Whatman No. 1 paper and the chromatogram developed using a chloroform–ethanol–glacial acetic acid 80:15:5 mixture as solvent. After drying the paper the alkaloid was located by immersion of the strip in Dragendorff's reagent[6]. Figure 1 shows a chromatogram comparing the chloro-compound with strychnine, strychnine hydrochloride and strychnine recovered by evaporation of the solvent from a chloroform solution, previously boiled for two hours. These data provided conclusive evidence that the chloro-compound, which may be formed during the assay, is distinct from strychnine and its hydrochloride.

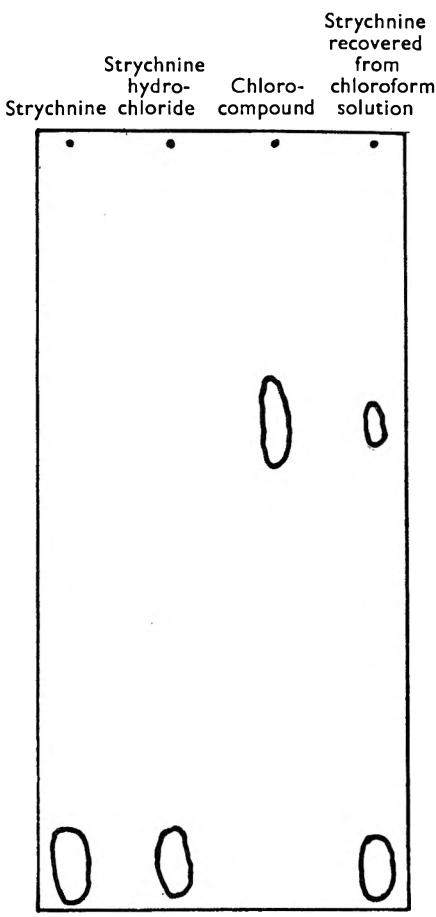


FIG. 1. Chromatogram using Whatman No. 1 paper and chloroform-ethanol-glacial acetic acid 80:15:5 mixture as solvent.

*Proposed Amendment to B.P. Assay of Strychnine Hydrochloride*

As a result of the work already described some modification of the B.P. assay procedure is necessary if the error due to the formation of the chloro-compound is to be eliminated. To avoid heating the chloroform solution we decided to explore the possibility of removing the alkaloid from the chloroform extract, obtained during the assay, by shaking with a known volume of standard acid, separating the acid layer and titrating the excess of acid with standard alkali. For this purpose the following experiment was repeated six times. Strychnine, weighing approximately 0·5 g. was dissolved in 25 ml. of 0·1 N sulphuric acid solution and the excess of acid titrated with 0·1 N sodium hydroxide solution using methyl red solution as indicator, the resulting assay figure being noted. The solution was then made alkaline by the addition of 5 ml. of sodium hydroxide solution and extracted with four portions (20 ml.) of chloroform, each extract being washed with two portions (10 ml.) of water.

The mixed chloroform extracts were shaken with 25 ml. of 0·1 N sulphuric acid solution and the mixture allowed to separate. The chloroform layer was removed and washed with three portions (15 ml.) of water, the washings being added to the acid extract. The mixed acid extract and washings were then titrated with 0·1 N sodium hydroxide solution using solution of methyl red as indicator. The results of these experiments are summarised in Table V.

TABLE V

SUMMARY OF RESULTS OBTAINED FOR THE AMENDED B.P. ASSAY

| Weight of strychnine taken g. | Assay expressed as per cent. $C_{21}H_{22}O_2N_2$ before extraction | Assay expressed as per cent. $C_{21}H_{22}O_2N_2$ after extraction | Per cent. recovery |
|---|---|---|---|
| 0·5368 | 99·4 | 99·0 | 99·6 |
| 0·5070 | 99·2 | 98·9 | 99·7 |
| 0·4581 | 99·2 | 99·1 | 99·9 |
| 0·4616 | 99·3 | 99·0 | 99·7 |
| 0·4222 | 99·1 | 98·9 | 99·8 |
| 0·4644 | 99·4 | 99·2 | 99·8 |

It is clear from these results that satisfactory assay figures can be obtained by the above procedure which eliminates the need to evaporate the chloroform extract containing the alkaloid. The main objection to the suggested modification is that chloroform being heavier than water causes the acid extraction and subsequent washing of the chloroform layer to be tedious and time consuming.

An alternative procedure, which affords satisfactory results, is to perform the B.P. assay but to titrate the final alkaloidal residue in glacial acetic acid solution with 0·1 N perchloric acid as shown in Table IV.

*Colorimetric Estimation*

For the determination of small quantities of strychnine, colorimetric methods, or more usually absorptiometric methods, are often employed after the alkaloid has been isolated by suitable means, which may involve extraction with chloroform. It therefore became of interest to ascertain whether the presence of the chloro-compound had any influence on the two colour reactions most widely used for quantitative work.

Colorimetric readings were obtained in the following manner for (1) strychnine, (2) a weighed amount of strychnine dissolved in chloroform and recovered from the solution, after standing overnight, by evaporation of the solvent and (3) the chloro-compound.

*Malaquin's reaction.* 0·2 g. of zinc amalgam activated before use by momentarily immersing in mercuric chloride solution was added to 5 ml. portions of the solutions containing 0·02 to 0·1 mg. of the substance, under examination, in 10 per cent. hydrochloric acid. The solutions were heated on a boiling water bath for nine minutes, cooled in running water for fifteen minutes and each treated with 1 drop of 0·1 per cent. sodium nitrite solution. The light absorption of each reaction mixture was then measured within 5 minutes with a Spekker absorptiometer using No. 547 Kodak filters and a blank, consisting of a reaction mixture

containing no alkaloid. The readings obtained are shown graphically in Figure 2.

*Ammonium vanadate reaction.* To 1 ml. portions of solution containing 0·04 to 0·2 mg. of substance, under examination, 5 ml. of reagent, containing 0·5 g. of ammonium vanadate in 100 ml. of 60 per cent. v/v sulphuric acid was added. After standing for two minutes 4 ml. of water was added and the light absorption of each reaction mixture

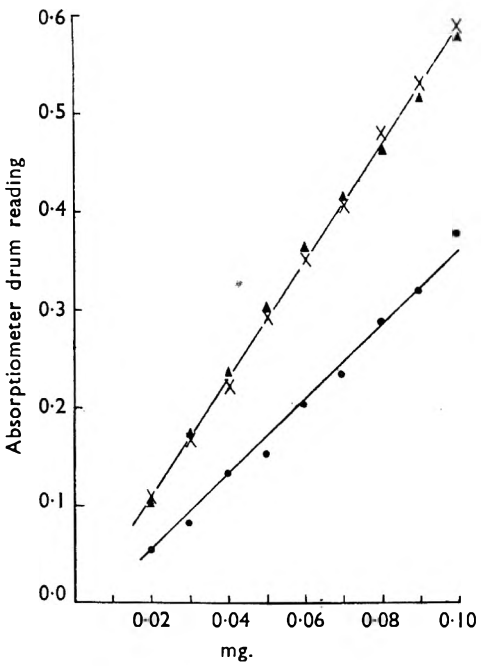


FIG. 2. Data for Malaquin colour reaction. ▲, strychnine; ×, strychnine recovered from chloroform solution; ●, chloro-compound.

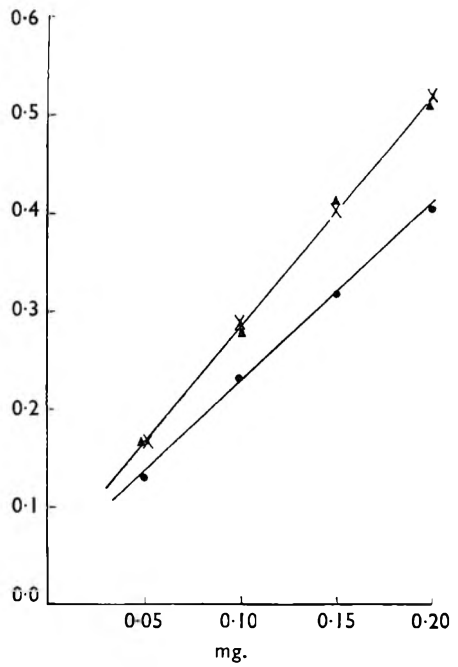


FIG. 3. Data for ammonium vanadate reaction. ▲, strychnine; ×, strychnine recovered from chloroform solution; ●, chloro-compound.

measured after 15 minutes with a Spekker absorptiometer using No. 547 Kodak filters and water as blank.

The readings obtained are shown graphically in Figure 3.

## DISCUSSION

The work described in this paper leaves no doubt that a reaction takes place between chloroform and strychnine when a solution of the alkaloid in this solvent is heated, even for the short period needed to remove the chloroform by evaporation during an assay. By prolonged heating of such solutions a chlorine containing reaction product separates from the solution and may be recrystallised from water. Paper chromatography has been used to establish that this chloro-compound may be formed in small amounts during the assay of strychnine salts by the normal procedure.

The reaction product was at first thought to be an addition compound containing one molecule of alkaloid and one of chloroform but analytical evidence was not in harmony with this view. Several samples on ultimate analysis gave analytical figures in close agreement but, so far, we have been unable to derive a satisfactory formula for the compound on the basis of the accepted structure of strychnine. It is noteworthy that part of the chlorine in the molecule is ionisable and part non-ionisable, while the product is dextro-rotatory in contrast with strychnine which has a laevo-rotation. Klemperer and Warren[7] have recently reported the isolation of strychnine dichloromethochloride, $C_{22}H_{23}O_2N_2Cl_3\frac{1}{4}H_2O$, from chloroform solutions of the alkaloid. It is probable that we have obtained the same substance, but the analytical figures reported by us are not in good agreement with the proposed formula and we prefer to postpone any statement regarding its structure until we have carried out further work.

Evidence has been provided that the formation of the chloro-compound causes a small but significant error in the assay of strychnine salts and preparations by assay procedures depending on weighing or titrating the alkaloid recovered from the chloroform extract by evaporation of the solvent. This error may be eliminated or reduced to negligible proportions by titrating the recovered alkaloid in glacial acetic acid with standard perchloric acid or by performing an extraction titration by shaking the chloroform extract with excess of standard sulphuric acid and titrating the excess of acid with standard sodium hydroxide.

The magnitude of the error will doubtless depend upon the conditions under which assays are performed in different laboratories and we are prepared to believe that some analysts will observe smaller errors than those reported in this paper. Nevertheless, we are convinced that the reaction between chloroform and the alkaloid constitutes a real source of error in the standard assay procedure and one which it is desirable to eliminate. In this connection the official assay of nux vomica, involving continuous extraction of the drug with ethanol-chloroform mixture, would seem to call for investigation.

Small quantities of strychnine are sometimes estimated colorimetrically using either the Malaquin reaction or the colour developed with ammonium vanadate in the presence of sulphuric acid. It has been shown that the presence of the chloro-compound does not affect the determination of strychnine by these methods, within the normal limits of experimental error.

## Summary

1. In the assay of strychnine salts by the standard procedure a discrepancy has been observed between the results obtained when the recovered alkaloid is weighed as strychnine and when it is titrated with standard acid.

2. A chlorine containing reaction product, for which analytical data are given, has been isolated from chloroform solutions of strychnine.

3. The formation of the chloro-compound interferes with the standard assay of strychnine salts.

4. Amendments to the standard assay procedure have been proposed with the object of eliminating the source of error.

5. The effect of the chloro-compound on the two most widely used colorimetric methods for the determination of strychnine has been investigated.

We wish to thank Mr. F. J. McMurray for the micro analyses reported in this paper.

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## DISCUSSION

The paper was presented by DR. G. E. FOSTER.

DR. D. C. GARRATT (Nottingham) asked whether the authors could have given attention to nux vomica and its preparations, because he felt sure that in their assay an empirical factor must have been worked out on a weight basis and not on the titration figure, and it would have been helpful to know if that correction factor was correct. In the assay of small quantities of strychnine it was always assumed that the weight obtained was that of pure strychnine, but he wondered whether there was not a balance of errors.

DR. F. HARTLEY (London) said that the preparation of the chloro-compound by refluxing the alkaloid with chloroform for some hours was not equivalent to the condition encountered in the assay procedure and asked whether the authors had assessed the rate at which the chloro-compound was formed.

DR. W. MITCHELL (London) suggested that the point could be emphasised with some advantage that the volumetric method gave low results and the gravimetric method high results, but neither gave the correct result. The authors had shown that non-aqueous titration gave an accurate result but suggested another method which was tedious. He suggested that it would be advantageous to adopt the convenient non-aqueous titration. In addition to the existing errors in the assay of nux vomica it was now necessary to take into account the loss due to the use of chloroform and he suggested that for initial extraction, benzene or some other solvent might be used instead of chloroform.

DR. A. H. BECKETT (London) said that the South African workers had based their conclusions on evidence which was completely unsatisfactory, but Dr. Foster had provided some rather interesting figures. The

equivalent weight given of 464·4 fitted exactly for one ionised chlorine atom and he hoped that the nitrogen figure 6·63 was an error and that it should have been 6·03.

DR. G. BROWNLEE (London) pointed out that should the chloro-compound prove to be quaternary in nature it would be expected to have very different pharmacological effects from those of strychnine.

DR. G. E. FOSTER, in reply, said that Mr. Caws was engaged in an investigation into the assay of nux vomica itself, and he hoped to publish a further paper. It was not necessary to reflux strychnine with chloroform to obtain the chloro-compound as they had recently found that this could be obtained by allowing a chloroform solution of strychnine to stand in the cold. The authors had not investigated the rate of reaction. It was true that non-aqueous titration gave the right result but that was because the factor used was that for strychnine. He had also queried the analytical figure for nitrogen, but the data had been checked and the printed figure was that actually found.

# STRUCTURAL REQUIREMENTS FOR ANALGESIC ACTIVITY IN ALKYLOXY-l-PHENYLETHYLAMINES AND SOME VIEWS ON ANALGESIC MECHANISM

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The complex pharmacological pattern characteristic of morphine, methadone and pethidine and their derivatives, suggests that these drugs have some common property when in contact with living tissues. The only readily discernible chemical similarity is possession of a phenylalkylamine unit of structure, a feature of a large number of drugs devoid of morphine-like properties. An attempt to trace the postulated common factor has been made by considering the structural requirements for morphine-like activity in 1-(*p-cyclo*hexyloxyphenyl)ethylamine[1], a substance more adaptable to systematic chemical studies than the better known therapeutic agents mentioned.

## Experimental

*Synthesis of materials.*—New materials were prepared by conventional methods as follows. Boiling ranges refer to air bath temperatures.

p-cyclo*Hexyloxybenzylamine.*—*p-cyclo*Hexyloxybenzoic acid[2], converted to the acid chloride and treated with aqueous ammonia gave the *amide*, that crystallised from benzene m.pt. 148° C. (77 per cent.). Found: C, 72·2; H, 7·7; N, 5·9. $C_{13}H_{17}O_2N$ requires C, 71·2; H, 7·8; N, 6·4 per cent. It was reduced by lithium aluminium hydride in boiling ether to give the above amine, b.pt. 130–140° C./2 mm. (80 per cent.). The *hydrochloride* crystallised from benzene-ethanol in prisms m.pt. 239° C. Found: C, 64·6; H, 8·5; N, 5·8. $C_{13}H_{19}ON$, HCl requires C, 64·6; H, 8·3; N, 5·8 per cent.

N - *Ethyl* - 1 - (p - cyclo*hexyloxyphenyl)ethylamine.*—1-(*p-cyclo*Hexyloxyphenyl)ethylamine gave the *acetamido derivative*, crystallised from light petroleum, m.pt. 85° C. (95 per cent.). Found: C, 73·3; H, 8·9; N, 5·4. $C_{16}H_{23}O_2N$ requires C, 73·6; H, 8·9; N, 5·4 per cent. It was reduced by lithium aluminium hydride in ether to the above amine. The *hydrochloride* crystallised from benzene-light petroleum in prisms, m.pt. 161° C. (88 per cent.). Found: C, 68·0; H, 9·1; N, 5·1. $C_{16}H_{25}ON$, HCl requires C, 67·7; H, 9·2; N, 4·9 per cent.

1-(p-cyclo*Hexyloxyphenyl*) - n - *propylamine.*—Cyclohexylation[3] of *p* - hydroxypropiophenone gave p-cyclo*hexyloxypropiophenone*, needles from light petroleum, m.pt. 63° C. (19 per cent.). Found: C, 77·5; H, 8·5. $C_{15}H_{20}O_2$ requires C, 77·6; H, 8·6 per cent. The *oxime*, needles from ethanol, m.pt. 115° C. (88 per cent. found: N, 5·8. $C_{15}H_{21}O_2N$ requires N, 5·7 per cent.) was reduced by sodium amalgam and acetic acid in methanol to the above amine, b.pt. 140–150° C./23 mm. (60 per cent.).

The *hydrochloride* crystallised from benzene-light petroleum in plates that fell to a powder when dried, m.pt. 167° C. Found: C, 66·5; H, 8·7; N, 5·2. $C_{15}H_{23}ON$, HCl requires C, 66·8; H, 8·9; N, 5·2 per cent.

NN-*Dimethyl*-1-(p-cyclo*hexyloxyphenyl*)*ethylamine*.—Prepared from 1-*p-cyclo*hexyloxyphenylethanol by treatment with phosphorus tribromide and reaction with dimethylamine. The amine b.pt. 180–185° C./16 mm. (48 per cent.) was converted to the *hydrochloride* and crystallised from benzene in prisms m.pt. 178° C. Found: C, 68·0; H, 9·2; N, 5·1. $C_{16}H_{25}ON$, HCl requires C, 67·7; H, 9·2; N, 4·9 per cent. The corresponding diethylamine could not be obtained as a crystalline derivative.

*Assay*.—Analgesic activity, taken as a measure of morphine-likeness, was sought by the method of D'Amour and Smith[4]. Groups of six albino rats, serving as their own controls received 1/10 to 1/5 of the approximate lethal dose for mice, and usually 20 mg./kg., intraperitoneally. The blacked tail tip was exposed to radiant heat and reaction times were measured before and at ten minute intervals for thirty minutes after the dose.

## Results

The amines described above caused only small changes in reaction times, within $\pm$ 10 per cent. of the control values, e.g., after the propylamine (10 mg./kg.; approximate lethal dose in mice, 105 mg./kg.), the mean control value of 9·8 $\pm$ 1·2 seconds fell to 9·3 $\pm$ 2 seconds at ten minutes and returned to 9·8 $\pm$ 2 seconds at twenty minutes after the dose.

The molecule of 1-(*p-cyclo*hexyloxyphenyl)ethylamine can be considered in four sections (I). The above and previous results have shown that

(*a*) Mono or dialkylation (Me, Et) of the amino group, or acetylation, gave inactive materials.

(*b*) Replacement of the methyl group by hydrogen or ethyl abolished activity.

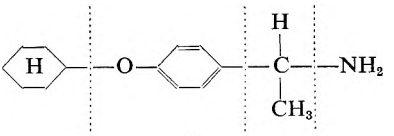
(*c*) Hydroxyl at position 3 reduced activity. Alkyl groups (Me, Et, $Pr^n$) at the same position abolished activity though depressant activity and toxicity increased.

(*d*) Only *cyclo*hexyl ethers were active. It had been shown however that moving the *cyclo*hexyl ether group to the 3 position had no influence on activity[1]. In summary, every feature of the molecule so far as examined, was essential for activity.

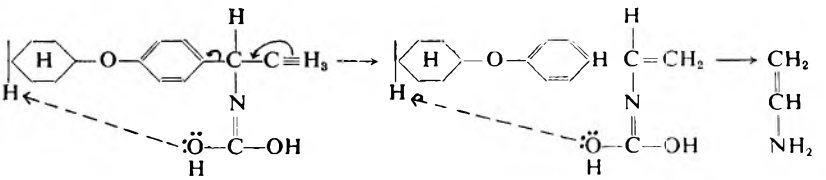
## Discussion

Evidence had been obtained[5] that the carbamic acid derived from (I) in the presence of carbon dioxide or bicarbonate could enolise. Formation of this species (II) appeared to require a *cyclo*hexyl group and a primary amino group. Since both these groups have been found to be essential for analgesic activity in the series, it seems justifiable to assume that II may be an intermediate active molecular species. Enolisation could not be linked to the essential methyl group.

Both II and methadone (III) have a carbon atom, adjacent to a benzene



I



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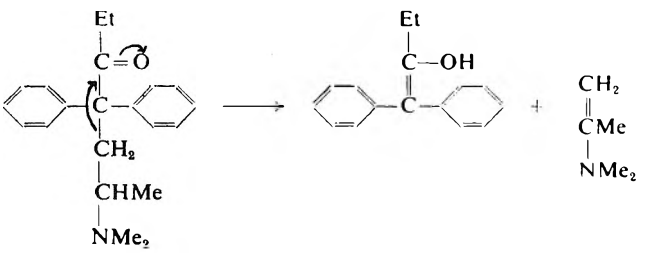
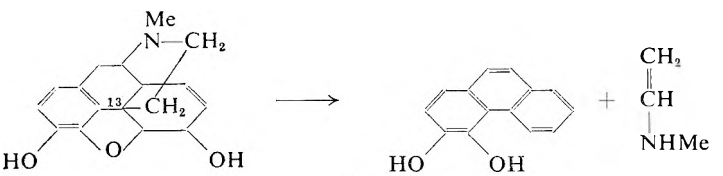
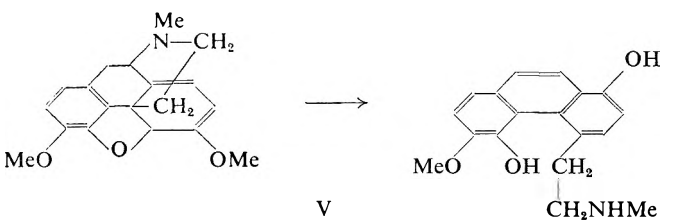


III



IV



V

nucleus, that occupies a central position in a system of unconjugated double bonds. In methadone a crossed conjugation can result if the alkamine chain be extruded as indicated. II can achieve a partial conjugation by similar electron transfer that cleaves the molecule to give a vinylamine, a process that requires both benzene nucleus and the methyl group. Removal of an ethanamine group from a benzene ring is known to occur during the oxidation of diiodotyrosine to thyroxine.

In morphine (IV), C(13) is probably under strain due to its position at the junction of four rings. The alkaloid is well known to extrude its

ethanamine chain under certain conditions, e.g., by heating with acetic anhydride, a process thought to be promoted by the drive towards conjugation (aromatisation) in the phenanthrene nucleus. Morphine can be regarded as a *gem*-substituted tetralin and extrusion of blocking groups during aromatisation can occur in this class of compound[6].

These three molecules appear to have a similarity in that an electron transfer around a carbon atom adjacent to a benzene nucleus can lead to extrusion of an ethanamine fragment to allow a fuller degree of conjugation in a system of double bonds. A similar consideration can apply to pethidine, e.g., if as seems likely, the piperidine ring is first cleaved to give products bearing analogy with methadone. Oxidative scission of piperidine derivatives is known to occur in animals[7]. The concept is difficult to apply to certain analgesics e.g., the dithienylbutenylamines, but it does offer a possible explanation for the inactivity of thebaine. Here, a major pathway of degradation (V), leads to thebenine. The initial process is probably the same as occurs with morphine, i.e., scission of the C(13)–C(15) bond, but the subsequent rearrangement gives a product that still retains the ethanamine chain.

The suggested degradations of II and III, perhaps foreign to concepts of organic chemistry, would be less surprising than some enzymically induced reactions. The stereospecificity of analgesic drugs and the proposals of Beckett[8] on overall shape of their molecules strongly suggest that an enzymic step is involved at some stage of analgesic mechanism.


## Summary

1. Modification of the structure of 1-(*p-cyclo*hexyloxyphenyl)ethylamine resulted in considerable loss of analgesic activity.

2. It is suggested that analgesic activity is a property of molecules that are capable under biological conditions, of extruding a vinylamine. This can be derived from an ethanamine structure associated with a strained carbon atom adjacent to a benzene nucleus.


## References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4. D'Amour and Smith, *J. Pharmacol.*, 1941, **72**, 74.
5. McCoubrey and Lynch, *J. Pharm. Pharmacol.*, 1956, **8**, 495.
6. Linstead and Thomas, *J. chem. Soc.*, 1940, 1127.
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## DISCUSSION

The paper was presented by THE AUTHOR.

DR. A. H. BECKETT (London) criticised the author's hypotheses of the reaction at the "analgesic receptor surface" for the following reasons: (1) Consideration of molecular models showed that hydrogen bonding between the enol oxygen and the 4-axial hydrogen of the *cyclo*hexane ring could not occur as shown in structure II. (2) The theory stated that

the driving mechanism for extrusion of the ethanamine group was derived from increase in conjugation effected thereby, but that was not true for compound II. In compound III the enol form would not be stable, the carbonyl group would not attract electrons from the amine-ethyl chain as shown, and the chemical evidence always showed cleavage between the carbonyl group and the quaternary carbon atom. It was illogical to use chemical arguments and then state that the degradations under biological conditions were foreign to concepts of organic chemistry.

DR. W. MITCHELL (London) asked the author whether he was suggesting that the analgesic action of morphine and the other compounds he had discussed was caused, directly or indirectly, by the ethanamine. If that were so, he was presumably suggesting that the rest of the molecule, acted simply as a carrier of the fragment.

DR. N. J. HARPER (London) said that Dr. McCoubrey found it difficult to explain the activity of the dithienyl type of analgesic. If the postulate concerning the degradation of methadone were correct, how did he explain the activity of acetylmethadols and also other analgesics such as the reversed esters of pethidine? The author offered an explanation of the inactivity of thebaine, but he suggested that the unsaturation in thebaine compared with morphine or diamorphine, was such that the nitrogen ring was labelised, e.g., the reaction of cyanogen bromide on morphine resulted in demethylation while in thebaine there was cleavage of the nitrogen ring with addition of the elements of cyanogen bromide. It seemed possible that in the metabolic process there was cleavage of the nitrogen ring with the result that the cleaved product might not fit the receptor site thought to be involved in the analgesic process.

DR. G. BROWNLEE (London) said that thebaine has, in an exaggerated degree the same excitatory actions as morphine and maybe the same receptor. One liked to think that morphine fitted an analgesic receptor site rather well but this was only one of the actions of morphine.

DR. MCCOUBREY in reply said that models could only be regarded as an approximation and should not be taken too seriously. He agreed that the apparent enolisation arises by some obscure mechanism and he would have preferred to avoid mention of hydrogen bonds, the last ditch of the organic chemist in difficulties. He admitted a seeming inconsistancy in using chemical concepts while at the same time disowning them. There is no exact knowledge of the mechanism of enzyme degradation and analogies based on chemical principles serve only as guides. The main hypotheses refers to electron redistribution by an unknown mechanism around a benzyl carbon atom with associated bond cleavage. He had suggested a possible driving force for certain examples. Dr. Mitchell had anticipated him in mentioning a carrier molecule concept and he was studying the theoretical probabilities of this.

# ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

## CHEMISTRY

### ALKALOIDS

***Lobelia salicifolia*, Alkaloids of.** E. Steinegger and F. Ochsner. (*Pharm. Acta Helvet.*, 1956, **31**, 97.) The alkaloids of *L. salicifolia* may be separated by paper chromatography. A new compound is given the name salicilobine, and appears to be derived from lobinanine by removal of methyl-ethyl ketone. Other compounds identified were nor-lobelanine, nor-lobelanidine, lobelanine, lobelanidine, (+)- and (−)-lobeline, decomposition products of the alkaloids, a new not-identified alkaloid, and a substance with an acrid taste. G. M.

### ANALYTICAL

**Deuterisation of Steroids and their Use in Isotope Dilution Analysis.** S. L. Jones, I. D. Robinson, B. H. Arison and N. R. Trenner. (*Analyt. Chem.*, 1956, **28**, 482.) This method was developed for the analysis of compound S (17-hydroxy-11-desoxycorticosterone) and compound F (17-hydroxy-corticosterone) in fermentation liquors during the microbial oxidation of compound F to compound S. Deuterium was introduced into the steroid molecule using a platinum catalyst and 70 per cent. deutero-acetic acid (prepared by the action of deuterium oxide on acetic anhydride). Known amounts of deutero compounds F and S are added directly to whole fermentation liquor, intimately mixed, then extracted, separated and purified. The dilution of deuterium in the isolated steroids is found, from which the original steroid content of the broth can be calculated. The steroids are separated by a countercurrent technique, and the deuterium is determined from the infra-red spectrum of the water formed on combustion of the steroids. D. B. C.

**Glyceryl Trinitrate Tablets, Analysis of.** G. Schwartzman. (*J. Assoc., off. agric. Chem.*, Wash., 1956, **39**, 254.) The infra-red absorption curve of glyceryl trinitrate in carbon disulphide is used for quantitative measurements at 6·05 and 7·90 $\mu$. Samples of tablets were extracted with carbon disulphide and the recorded spectra of the sample and a standard from 2–15 $\mu$ were compared to ascertain the identity of the sample; the baseline absorbance of each was then determined at 7·90 $\mu$. Five samples of commercial nitroglycerin tablets were analysed by the proposed method and by the U.S.P. XV assay; the results show good agreement. R. E. S.

**Griseofulvin in Fermentation Samples, Determination of.** G. C. Ashton and A. P. Brown. (*Analyst*, 1956, **81**, 220.) A physico-chemical procedure for the determination of griseofulvin is described involving extraction of whole-broth samples with butyl acetate and measurement of the ultra-violet spectrophotometric absorption of the extract. Irrelevant ultra-violet absorption in the extract is allowed for mathematically by a correction procedure which is described. Results obtained by an isotope dilution method based on $^{36}Cl$ griseofulvin are reported by Ashton (*ibid.*, 228) and are compared with those obtained by the spectrophotometric method. D. B. C.

**Morphine in *Papaver somniferum*, Ion Exchange Separation of, before Assay.** C. H. van Etten, F. R. Earle, T. A. McGuire and F. R. Senti. (*Analyt. Chem.*, 1956, **28**, 867.) In the processing of morphine from poppy plants, the following rapid and accurate method was used:—An aqueous extract was prepared by a suitable extraction process. A sample containing 5- to 20-mg. of morphine in 5- to 50-ml. was passed through a 5 cm. column of Dowex 50 X 1 cation exchanger in the H-form, washed with 5 to 10 ml. of water and eluted with 50 ml. of 0·5N ammonium hydroxide. The eluate was then passed through an anion exchange 6 cm. column of Dowex 1 X 1 Cl in the OH-form which retained the morphine and all the other ampholytes. The column was washed with three × 5 ml. of water and eluted with 50 ml. of 0·3N acetic acid. This eluate was passed through a cation developer 5 cm. column in the Na-form (Dowex 50 X 1 Na). This column was then eluted with a borate buffer of pH 8·6 until the effluent reached this pH, then 30 ml. more buffer was passed through. The morphine-containing fraction was then eluted with 225 ml. of a borate buffer pH 9·4. This eluate, after acidification with 3N hydrochloric acid, was concentrated on a steam bath and then diluted to 50 or 100 ml. From this, samples containing 0·1 to 2·0 mg. of morphine were analysed colorimetrically by the colour produced with nitrous acid, or by ultra-violet absorption. Larger amounts could be isolated and estimated by titration. The average value for recovery of 20 mg. morphine was 98 per cent. with a standard deviation of 1·9. D. B. C.

**Organic Nitrogen, Ampoule Combustion—Isotope Dilution Technique for.** S. L. Jones and N. R. Trenner. (*Analyt. Chem.*, 1956, **28**, 387.) The principle of isotope dilution is used in this method which can estimate with precision small quantities of elemental nitrogen in organic compounds. The sample (trace, or tracer and unknown) is converted into nitrogen gas by sealing with an excess of cupric oxide in an evacuated ampoule of high-silica glass and heating in a furnace to complete combustion. The ampoule is opened in a previously evacuated ampoule breaker and the combustion gases, after absorption of carbon dioxide and water, are passed into a mass spectrometer where the isotope ratio of the nitrogen is determined. The sample size can be well under 1 mg. An error of ± 0·5 per cent. may be contributed by the background in the mass spectrograph, while the residual nitrogen from the copper oxide and ampoule may contribute another 0·5 per cent. error. Apart from this the accuracy depends upon the weighing of the small samples. D. B. C.

**Phenobarbitone, Argentimetric Potentiometric Titration of.** J. I. Bodin. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 185.) The method is a modification of a dead-stop end-point method, in which the uncertainty in the end-point potential is eliminated by determining the potential of a standard blank solution just before the titration of each sample. This is necessary because the end-point potential varies from time to time according to the condition of the electrodes. A silver indicator electrode is placed in the solution to be examined and the solution connected by a salt bridge to a saturated calomel electrode. Prior to titration, the potential of a solution containing 10 ml. of ethanol (95 per cent.), 50 ml. of 3 per cent. sodium carbonate solution and 1 ml. of 0·01N silver nitrate with water to 100 ml. is determined. The sample is dissolved in the same solvent (without the silver nitrate) and titrated to the potential of the blank. Good accuracy is reported by this method which is not affected by the usual amounts of excipients, although stearates should be removed before titration, and correction is necessary in the presence of ethanol or hexamine. G. B.

**Sugars and Related Substances, Colorimetric Method for.** M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith. (*Analyt. Chem.*, 1956, **28**, 350.) Simple sugars, oligosaccharides, polysaccharides, and their derivatives including the methyl ethers with free or potentially free reducing groups, give an orange-yellow colour when treated with phenol and concentrated sulphuric acid. The reaction is sensitive and the colour is stable. By the use of this phenol-sulphuric acid reaction, a method of estimating 10–70 $\mu$g. amounts of sugars and related substances has been developed. In conjunction with paper partition chromatography, a synthetic mixture of sugars can be analysed, and the method is useful for determining the composition of polysaccharides and their methyl derivatives. D. B. C.

**Vitamins $D_2$ and $D_3$ in Pure Solution, Estimation of.** D. H. Laughland and W. E. J. Phillips. (*Analyt. Chem.*, 1956, **28**, 817.) The method is based upon the formation of a coloured reaction product when the vitamins are treated with furfural and sulphuric acid under carefully controlled conditions. The method is applicable to binary mixtures and to samples containing as little as 15 $\mu$g. of total vitamin D, regardless of the relative abundance of each. The details are as follows:—Place a 2·0 ml. aliquot (not less than 15 $\mu$g. of total vitamins) of an ethanol solution of the vitamins in a 50 ml. centrifuge tube. Add 1·0 ml. of furfural reagent (0·0046 per cent. in 95 per cent. ethanol) and cool the tube by immersion in a mixture of ethanol and dry ice in a Dewar flask. Stir with a fine stream of nitrogen and maintain the temperature at 13° $\pm$ 5° C. by raising or lowering the tube. Add 7·0 ml. of concentrated sulphuric acid dropwise at about 1 ml. per minute. Allow to come to room temperature and determine the absorption curve 20 minutes after commencing to add the acid. Both forms of the vitamin complex absorb at 490 m$\mu$ while that of vitamin $D_2$ shows a subsidiary peak at 565 m$\mu$, and the absorption of the vitamin $D_3$ complex is neglegible at this wavelength, so that assays of mixtures can be based on the ratio of absorbancies at these wavelengths. Of substances structurally related to the vitamins, only 7-dehydrocholesterol and ergosterol interfere seriously, and all show different absorption curves from those of the vitamin complexes. D. B. C.

# BIOCHEMISTRY

## BIOCHEMICAL ANALYSIS

**Cobalt in Animal Tissues, Estimation of Trace Quantities of.** R. G. Keenan and J. F. Kopp. (*Analyt. Chem.*, 1956, **28**, 185.) Two spectrochemical methods for the determination of submicrogram quantities of cobalt in animal tissues have been devised which are more sensitive and less subject to the effect of interfering elements, notably iron, than chemical methods. One method is applied directly to the ash of tissues containing at least 0·025 $\mu$g. of cobalt per g. of fresh tissue in excess of that present in normal tissue and was applied to animals exposed to cobalt in the course of an investigation of the toxicity of this element. The other method was developed for the determination of cobalt in normal tissues. This employs the principle of preliminary chemical concentration with 1-nitroso-2-phenol as a complexing agent. The next stage in both methods is the collection of the cobalt in a mixture of aluminium oxide, lithium chloride and graphite which is loaded into the specially prepared crater of a spectroscopically pure graphite electrode of standard dimensions. The

concentration of the cobalt is calculated from standard curves based on photographs of the spectra of known concentrations under identical conditions. For the direct method, the error is ± 10 per cent., while that for the cobalt concentration method is − 6 per cent. D. B. C.

**Hæmoglobin in Plasma, Spectrophotometric Determination of Total.** K. B. McCall. (*Analyt. Chem.*, 1956, **28**, 189.) All hæmoglobin is first converted to methæmoglobin; the absorbance of this solution is then evaluated before and after a small amount of cyanide is added to convert all the methæmoglobin to cyanmethæmoglobin. The change in absorbance observed is directly proportional to the total hæmoglobin and is calculated directly. The method offers the advantages of simplicity, stable reagents, and the production of stable colours with an acceptable degree of precision. D. B. C.

**Nicotine in Urine, Nephelometric Determination of.** M. Mokranjac, S. Radmić and E. Galijan. (*Acta Pharm. Jug.*, 1955, **5**, 115.) To 50 ml. of urine add an excess of sodium hydroxide solution and distil until only a few ml. remains. Add to the distillate 25 ml. of water and redistil. Distil once more and extract the distillate with 3 successive quantities of 15 ml. of ether. Mix the ethereal solutions, add 5 drops of 0·5 per cent. hydrochloric acid to prevent loss of nicotine by volatilisation and evaporate the ether. To the residue add 5 drops of modified Sonnenschein reagent (phosphomolybdate in nitric acid) and dilute to 3 ml. with water. Compare the optical density of the suspension obtained with that of suspensions prepared from known quantities of standard nicotine solution in the same way and hence calculate the quantity of nicotine in the urine. Samples containing 25 to 500 μg. of nicotine may be assayed by this method. G. B.

**Vitamin A, Slope-Ratio Liver-Storage Bioassay for.** S. R. Ames and P. L. Harris. (*Analyt. Chem.*, 1956, **28**, 874.) Five groups of depleted rats were supplemented as follows: two levels of the reference standard (1000 and 2000 units), two similar levels of test material, and a negative-control group. The relative potency is determined by the ratio of the slopes of the two linear dose-response lines. The liver-storage of vitamin A showed an essentially linear response over a dose range of 500 to 10,000 units. Direct comparison of the results of this method and that of growth response showed no significant difference. After depletion and feeding, details of which are given, the animals are killed and the entire livers are removed, blotted, weighed and stored at − 15° C. until analysed. Each rat liver is ground in a mortar with anhydrous sodium sulphate until dry, extracted with peroxide-free anhydrous ether, and after removal under nitrogen of the ether from an aliquot part of the extract, the intensity of the blue colour formed on the addition of antimony trichloride is measured in a suitable colorimeter using a 620 mμ filter, and the result estimated from a standard curve. Full details of statistical analysis are given. The standard error of the assay is less than ± 10 per cent. D. B. C.

## PHARMACY

### NOTES AND FORMULÆ

**Agars, Determination of the Grade Strength of.** N. R. Jones. (*Analyst*, 1956, **81**, 243.) The proposed method is based on a determination of the percentage of agar necessary to produce an agar-water jelly of a given strength when prepared under standard conditions. The standard jelly strength chosen is

one of 75 g., for a deflection of 20° on the F.I.R.A. jelly tester (i.e., that of the Food Industries Research Association obtainable from Messrs. H. A. Gaydon and Co. Ltd., Croydon, Surrey). The jelly tests are made at two concentrations of agar (0·5 and 1·0 per cent.) and the concentration required to produce a jelly strength of 75 g. is found by interpolation. The grade strength is conveniently expressed as the number of grams of jelly of the standard strength obtainable from 1 g. of agar. D. B. C.

## PHARMACOLOGY AND THERAPEUTICS

**Acetyl-Digitoxin, Clinical Experience with.** M. Goldfarb, M. C. Thorner and G. C. Griffith. (*Amer. J. med. Sci.*, 1956, **231**, 186.) Acetyl-digitoxin is a new cardiac glycoside prepared from *Digitalis lanata* by removal of one glucose molecule from lanatoside A. It has been studied in 82 patients over periods up to eight months. It acts quickly and it is completely absorbed from the gastrointestinal tract. In congestive heart failure it decreases heart size and rate and produces a marked diuresis. Initial digitalization was obtained with a total dose of 1·8–2·4 mg. (0·6–0·8 mg in three four hour doses), and the maintenance dose was from 0·1–0·2 mg. daily. Signs of toxicity were seen in one-fifth of the patients, untoward effects being nausea, vomiting, diarrhœa, blurred vision, headache and premature ventricular contractions. These effects are prevented by decreasing the dose. It is suggested that acetyl-digitoxin is indicated in all forms of congestive heart failure, in uncontrollable atrial fibrillation, paroxysmal tachycardia and atrial flutter. G. F. S.

**Angiotonin and (—)-Noradrenaline, Relation of, to Essential Hypertension.** S. E. Greisman. (*J. exp. Med.*, 1956, **103**, 477.) If a humoral pressor substance is responsible for essential hypertension in man, one of its characteristics should be the ability to constrict the arteriolar bed sufficiently to increase the total peripheral resistance, but not sufficiently to reduce the cutaneous blood flow. This hypothetical pressor substance should also be capable of producing a generalised systemic arteriolar constriction without inducing significant constriction or decreased blood flow in a cutaneous capillary bed such as the nailfold. The purpose of this work is to determine the reaction of the nailfold bed in persons made hypertensive by the infusion of angiotonin and to compare this reaction with that seen in persons with hypertension induced by noradrenaline and in persons with essential hypertension. Such observations indicate that angiotonin, unlike noradrenaline, is capable of raising the systemic arterial blood pressure without inducing sustained ischæmia of the nailfold capillary bed. Evidence was also obtained that the nailfold bed of persons made hypertensive by the intravenous infusion of angiotonin exhibited a hyper-activity to circulating noradrenaline, similar to that found in patients with essential hypertension. From these results it seems that angiotonin is a substance which possesses the properties required of the hypothetical substance of essential hypertension. M. M.

**Cerebral Tissue Extracts, Spasmolytic Effects of.** O. C. Forbes. (*Nature, Lond.*, 1956, **177**, 893.) Previous work has shown that contractions of the guinea-pig ileum caused by acetylcholine or by adenosine triphosphate can be antagonised with extracts of rat brain. Using an extract of dried red-cell stroma, there was relaxation of the acetylcholine responses but not of the adenosine triphosphate contractions. An investigation was then carried out into the possible presence of a relaxing agent other than choline esterase in brain extracts. An extract of cow brain consisting of crude sphingosine bases

was found to abolish the acetylcholine and the adenosine triphosphate responses of the guinea-pig ileum, similarly to whole brain extracts. Using the Trendelenburg preparation, this extract abolished the peristaltic movement of the ileum. A more purified fraction (triacetylsphingosine) showed qualitatively similar effects. Thus sphingosine and allied bases in cerebral tissue may exert a powerful spasmolytic action *in vitro*. M. M.

**Cortisone, Effect of, on Blood.** T. Nicol and D. L. J. Bilbey. (*Nature, Lond.*, 1956, **177**, 524.) The phagocytic activity of the reticulo-endothelial system is depressed during the first two weeks of cortisone treatment, activity returning to normal levels during the third and fourth weeks of treatment. Changes have now been shown to occur in the blood from guinea-pigs given 10 mg. cortisone daily by intramuscular injection for five weeks. Blood was removed by heart puncture before the start of treatment and thereafter at the end of each week. Blood films were stained with Giemsa stain. During the first two weeks of treatment the degree of polychromasia and anisocytosis increased above normal levels. At the end of the second week immature red cells were present in greater numbers, although the red-cell count remained unaltered; the hæmoglobin-level fell from 81 to 70 per cent. The total leucocyte count dropped below normal during the first three weeks of treatment, but rose above normal during the fourth and fifth. The differential white cell count showed a sustained fall in lymphocytes during cortisone administration; pseudo-eosinophils on the other hand gradually increased in numbers. G. P.

**Cortisone, Effect of, on the Reticulo-endothelial System.** T. Nicol and R. S. Snell. (*Nature, Lond.*, 1956, **177**, 430.) In a previous communication (*Nature, Lond.*, 1954, **174**, 554) the authors have shown that cortisone depressed the activity of the reticulo-endothelial macrophages, particularly in the spleen, and suggested that the lowered resistance to infection shown by patients undergoing cortisone treatment resulted from decreased macrophage activity. In the present communication the results have been extended in dose range of cortisone and duration of treatment. 79 male guinea-pigs received daily injections of trypan blue subcutaneously for six days before being killed by chloroform. Seven of the animals were used as controls, the others received daily intramuscular injections of 5, 10 or 25 mg. of cortisone for a period of 1, 2, 3 or 4 weeks. The uptake of dye by the macrophages of the spleen, liver, and lymph nodes was markedly lower in the animals treated for one to two weeks with cortisone than in the control animals. This depressant effect was seen earlier in animals given the larger doses of cortisone. After cortisone treatment for three or four weeks, dye uptake by the macrophages was the same in treated animals as in the controls. This suggests that patients should be specially protected from intercurrent infection during the early stages of cortisone treatment. G. P.

**Cortisone, Effect of, on the Serum Gamma-Globulin.** R. S. Snell and T. Nicol. (*Nature, Lond.*, 1956, **177**, 578.) This paper deals with the effect of cortisone on the antibody level in the serum. The serum $\gamma$-globulin is taken as a measure of the antibody level since most antibodies are found in association with the $\gamma$-globulin fraction of the serum protein. The estimation was made by first separating the $\gamma$-globulin by paper electrophoresis, treating the paper with dyes and estimating the optical density of the protein dye complex by means of a photoelectric cell. By this means the percentage of $\gamma$-globulin in relation to the total serum protein could be assessed. Male guinea-pigs were used.

Blood samples were taken from each animal by heart puncture and the $\gamma$-globulin level estimated as described above. Each animal was then given a daily dose of 10 mg. of cortisone intramuscularly for five weeks. Blood samples were taken at weekly intervals. It was found that in all the animals there was a marked reduction of the $\gamma$-globulin level during the first two weeks of the injections and that subsequently it remained at a low level. These results emphasize the profound depression of the body defences by cortisone and the great necessity to protect patients from intercurrent infection especially during the early stages of cortisone therapy. M. M.

**Dichloralphenazone and Chloral Hydrate, Comparison of.** W. B. Rice and J. D. McColl. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **43**, 137.) Chloral hydrate and dichloralphenazone were tested for acute toxicity in mice, for chronic toxicity and hypnotic activity in rats, for onset and duration of hypnotic activity in guinea-pigs, for analgesic action in mice and for antipyretic action in rabbits. The two compounds were found to be similar in activity and toxicity when considered on the basis of chloral content. Slightly more chloral was required to produce hypnosis when used in the form of dichloralphenazone, but the ratio of lethal and hypnotic doses was approximately the same. The analgesic effect of dichloralphenazone appeared to be due mainly to the phenazone content. Dichloralphenazone has the advantage of ease of handling, being a stable crystalline compound, free from unpleasant odour and taste, and may be used in place of chloral hydrate. G. B.

**Dimethylaminoethanol, Synthetic Esters of, Exhibiting Positive Inotropic Cardiac Activity.** F. C. Uhle, B. A. Mitman and O. Krayer. (*J. Pharmacol.*, 1956, **116**, 444.) The erythropleum alkaloids, which, like the cardiac glycosides, improve the competence of the failing mammalian heart, have been shown to be $\beta$-dimethylaminoethyl or $\beta$-methylaminoethyl esters of $\alpha,\beta$-unsaturated tricyclic hydroaromatic acids of otherwise unknown structure. While hydrolytic cleavage of the alkaloids leads to totally inactive carboxylic acids, the alkanolamines still retain the qualitative actions of the parent alkaloid, although greatly diminished in potency. Twenty-six esters of one of these alkanolamines, dimethylaminoethanol, with aliphatic, aromatic and $\alpha,\beta$-unsaturated carboxylic acids were prepared and tested for this activity in conditions of failure of the Starling heart-lung preparation of the dog, failure being induced by sodium pentobarbitone. Only the difunctional esters of succinic, glutaric, adipic, and pimelic acids had positive inotropic activity—of the order of five to ten times that of the parent alkanolamine. The esters of azelaic and sebacic acids had negligible activity; the esters of some of the other acids, particularly of complex polynuclear acids, caused irregularities of rate and rhythm. G. P.

**Ethylmethyl-*iso*octenylamine (EMOA), a New Parasympathetic Ganglionic Blocking Agent.** E. G. Pardo, I. Méndez, R. Vargas, J. Cato and J. Laguna. (*J. Pharmacol.*, 1956, **116**, 377.) Of the tertiary amine derivatives of Octin (methyl-*iso*octenylamine) the *N*-ethyl analogue (EMOA) was a more potent spasmolytic agent on the isolated intestine of the rabbit than were Octin, ephedrine, papaverine and diethylmethyl-*iso*octenyl amine (DEMOA). In dogs, stimulation of gastric secretion by histamine was inhibited after intraduodenal or intravenous administration of EMOA; minimal intraduodenal doses having this action had no effect on blood pressure. EMOA had a vasodepressor action in anæsthetised cats, the magnitude and duration

of the fall depending upon dose. The cardiac response to vagal stimulation was blocked by doses causing an appreciable fall in blood pressure; the vasodepressor action of acetylcholine was, however, unaffected. Also, the normal fall in pressure with tetraethylammonium (TEA) or hexamethonium was absent after these doses of EMOA, in some cases TEA causing a rise in pressure after EMOA; on the other hand, TEA had no effect on the action of EMOA. Neither neuromuscular transmission nor transmission through the superior cervical ganglion in the cat was blocked by relatively large intravenous doses of EMOA, but the compound caused a moderate, sustained contraction of the nictitating membrane. Cardiac output in the cat heart-lung preparation was greatly reduced, with only a slight decrease in heart rate. Toxicity of the drug in mice by both oral and intraperitoneal routes was relatively low. There was a slight stimulation of the central nervous system with the compound. The effects of EMOA were interpreted in the main as a selective action on parasympathetic ganglia; in addition, the drug had a direct action on the heart and slight sympathomimetic activity. G. P.

**Ethyl 4-Phenylpiperidine-4-carboxylates, *N*-Substituted, Analgesic Action of.** R. A. Millar and R. P. Stephenson. (*Brit. J. Pharmacol.*, 1956, **11**, 27.) A series of pethidine derivatives, in which the *N*-methyl group has been replaced by a tertiary amino alkyl group, has been tested for analgesic activity in rats by the tail pressure method of Green and Young. The best compound was morpholinoethylnorpethidine which was three to seven times more active than pethidine. Substitution of S for O in the morpholino ring reduced activity to one-third, and lengthening or shortening the chain of C atoms linking the nitrogen atoms of the two rings considerably reduced activity. Branching also reduced activity. Unlike pethidine the compound did not induce convulsions in mice, but it caused a Straub tail reaction. In spite of its higher analgesic activity the toxicity of this compound was not greater than pethidine to mice. Its analgesic actions were antagonised by nalorphine. G. F. S.

**Hydroxy*iso*phthalic Acids, Antipyretic and Analgesic Properties of.** H. O. J. Collier and G. B. Chesher. (*Brit. J. Pharmacol.*, 1956, **11**, 20.) 4-Hydroxy-*iso*phthalic acid (4-HIPA) and 2-hydroxy*iso*phthalic acid (2-HIPA), byproducts of the Kolbe-Schmitt process for the manufacture of salicylic acid, have been shown to have an antipyretic action, at least as great as aspirin, in rabbits treated with Proteus pyrogen. Single large intraperitoneal doses of both compounds raised the pain threshold of rats to pressure applied to the tail, and the analgesic activities were greater than aspirin but less than salicylamide and codeine. Analgesia was not accompanied by loss of righting reflex, drowsiness or other visible side effects. The analgesic activity of 4-HIPA was not antagonised by nalorphine, but it was potentiated by codeine, methylpentynol, pentobarbitone and thiopentone. 4-HIPA had a very slight local anæsthetic action. Acute experiments in rats showed that both 2-HIPA and 4-HIPA were less toxic than codeine, aspirin and salicylamide. Subacute and chronic toxicity tests of 4-HIPA showed it to be similar in toxicity to aspirin. 0·2 per cent. of 4-HIPA in the diet of young rats produced only a very slight depression of growth. G. F. S.

**5-Hydroxytryptamine, Estimation of, in the Presence of Adrenaline.** J. D. Garven. (*Brit. J. Pharmacol.*, 1956, **11**, 66.) The isolated œstrous uterus of the rat contracts to 5-HT but the response is inhibited by adrenaline and noradrenaline, which may be present in some tissue extracts making the assay results

too low. This paper describes the use of mushroom juice, which contains a polyphenoloxidase enzyme, for the elimination of the interference. The tissues were double extracted with acetone which leaves substance P in the insoluble residue. The extracts were filtered and evaporated to dryness at 30 to 35° C. under reduced pressure. One ml. of water was then added to the residue and the solution extracted twice with light petroleum to remove lipids. The aqueous residue was evaporated to dryness under reduced pressure. For the assay, the residue was dissolved in water and treated with a prepared mushroom juice for forty minutes at room temperature. The solutions were then assayed for 5-HT activity in the usual way. Using this method the 5-HT content was determined in a whole series of rabbit tissues. Significant amounts of 5-HT were found in the spleen, various areas of the gut mucosa, blood and serum. The hypothalamus, liver and bone marrow showed smaller amounts. Other tissues (lung, thyroid, pancreas and diaphragm) contained little 5-HT and none was detected in skeletal muscle, nerve and adrenals. The specificity of the extract activity was determined by treatment with lysergic acid diethylamide. G. F. S.

**Iproniazid, Mechanism of Drug Potentiation by**. J. R. Fouts and B. B. Brodie. (*J. Pharmacol*, 1956, **116**, 480.) Iproniazid (Marsilid or 2-*iso*propyl-1-*iso*nicotinyl hydrazine), a substance with practically no sedative action, prolonged the hypnotic activity of hexobarbitone in mice by inhibiting its metabolic degradation; mice given 50 mg./kg. of iproniazid intraperitoneally in addition to a hypnotic dose of hexobarbitone slept about three times as long as controls given only the barbiturate. The rate of metabolism of the hexobarbitone was estimated by homogenising animals killed either twenty minutes after administration of the drugs or at the point of awakening; at twenty minutes the concentration of hexobarbitone was twice as high in the iproniazid-treated mice as in mice receiving the hexobarbitone alone. However, at the point of awakening, the concentrations of barbiturate were approximately the same in treated and untreated animals. Also, once awakened, the mice could not be returned to sleep with large doses of iproniazid, confirming the indirect action of the prolonging agent. Iproniazid inhibited the oxidative enzyme systems in liver microsomes which oxidise the side chain of hexobarbitone, dealkylate aminopyrine, deaminate amphetamine and hydroxylate acetanilide. Its action is therefore similar to that of β-diethylaminoethyl diphenylpropylacetate (SKF 525-A) and 2:4-dichloro-6-phenylphenoxyethyl diethylamine (Lilly 18947). The way in which the inhibition is occasioned is unknown, but is presumably not due to an interchange of the iproniazid with the nicotinamide moiety of di- or tri-phosphopyridine nucleotide. All three inhibitors, despite dissimilarity of structure, probably act by the same mechanism. G. P.

**Lysergic Acid Diethylamide, Tolerance to the Pyretogenic Action of**. J. W. Gogerty and J. M. Dille. (*J. Pharmacol.*, 1956, **116**, 450.) Tolerance to administration of lysergic acid diethylamide (LSD-25) has been reported in patients after seven days' treatment with the drug (H. F. Isbell and others, *Fed. Proc.*, 1955, **14**, 354). A similar tolerance to the pyretogenic effect of LSD-25 developed in rabbits with daily administration of 50 μg./kg. intravenously for a period of four to five days. Complete abolition of the response was not obtained in this time, but the effect was greatly reduced. The tolerance persisted for up to nine days after withdrawal of the treatment schedule. G. P.

**Nystatin; Effect on Growth of *Candida albicans***. A. J. Childs. (*Brit. med. J.*, 1956, **1**, 660.) This investigation was carried out to see if the simultaneous administration of nystatin to patients receiving tetracycline would have any

effect on the overgrowth of *C. albicans*. The patients studied were 50 males, whose ages ranged from 12 to 81 years, suffering from pneumonia. They had not been treated with adequate chemotherapy prior to admission. The patients were allocated alternately to treatment either with tetracycline alone or with tetracycline plus nystatin. Both groups were given tetracycline orally in a dosage of 0·25 g. four-hourly for 48 hours, after which the dose was reduced to 0·25 g. six-hourly for a further 3 days. In the second group, in addition to the tetracycline, nystatin was given orally, the dose being one tablet, containing 500,000 units, eight-hourly. The frequency with which *C. albicans* occurred in throat swabs, rectal swabs and sputum was observed before, during and after treatment in both groups. Examination of admission specimens showed that *C. albicans* was present in 26 per cent. of all rectal swabs, 24 per cent. of all throat swabs, and 38 per cent. of all sputa. Treatment with tetracycline caused an increase in the number of specimens from which *C. albicans* could be isolated; there was a gradual rise up to the seventh day in hospital. When nystatin was added to the tetracycline the culture results were not uniform but there seemed to be a tendency towards lower yields. In rectal swabs there was a definite trend towards elimination of heavy growths. In throat swabs the effect was less obvious, though no marked rise occurred. So far as the sputum was concerned the two treatment groups seemed to be similar. These results are in keeping with the view that nystatin does not appear in the blood stream in adequate concentration and that it might be ineffective in the treatment of systemic infection. S. L. W.

**Oxytocin, Clinical Trial of.** H. H. Francis and W. J. A. Francis. (*Brit. med. J.*, 1956, **1**, 1136.) Synthetic oxytocin (Syntocinon containing 10 international units per ml.) has been compared with purified natural oxytocin (Pitocin) in thirty-one patients near term or in labour. The solutions were administered by continuous drip in 5 per cent. dextrose solution at strengths of 2·5, 5, or 10, I.U. per litre. The dose was regulated by the rate of infusion according to the uterine response. Uterine contractions were recorded by a Lorand tocograph, observations being restricted to the first and second stages of labour. The results showed Syntocinon to be strongly oxytocic in all cases and the type, amplitude and frequency of uterine contractions to be indistinguishable from the same dose of Pitocin. The synthetic product was free from undesirable side effects, except for a slight pressor activity too slight to contraindicate its use in therapeutic dosage. G. F. S.

**Oxytocin, Synthetic.** M. N. Bainbridge, W. C. W. Nixon, H. O. Schild and C. N. Smyth. (*Brit. med. J.*, 1956, **1**, 1133.) Synthetic oxytocin (Syntocinon), standardised biologically to be equal to the international standard preparation, has been studied clinically on the human uterus. Comparative assays were made with oxytocin, using the action on the corpus and cervix uteri of patients undergoing therapeutic abortion, the action on the uterus of patients in labour and the action upon the uterus in post-partum patients on the second or third day after delivery. The results showed that there were no qualitative or quantitative differences between the two preparations. G. F. S.

**Sodium 3:5-Diacetamido-2:4:6-triiodobenzoate (Hypaque Sodium), a New Urographic Contrast Medium, and Related Compounds, Toxicity of.** J. A. Hoppe, A. A. Larsen and F. Coulston. (*J. Pharmacol.*, 1956, **116**, 394.) The toxicity of a series of 3:5-diacylamino-2:4:6-triiodobenzoic acids was determined for the mouse, rat, rabbit, cat and dog. The least toxic of the series

(ABSTRACTS—*continued on page 816.*)

# BOOK REVIEWS

*KOMMENTAR ZUR PHARMACOPOEA HELVETICA, V.* Supplement I and II, pp. xi + 316 (including Index). Schweizerischer Apothekerverein, Zurich. 1956. Sw.Fr.32.00.

The volume is a complete new edition of earlier volumes published in 1947 and 1949 as commentaries on the Swiss Pharmacopœia V and its supplement. Publication of the Supplement II to the Swiss Pharmacopœia in 1955 brings the total number of substances official for the first time in the two supplements to about 160, and included many alterations and extensions of existing monographs. The present volume, which in some ways might be compared with the *Extra Pharmacopœia*, provides a commentary on these new and revised monographs, and has been compiled by a group of experts, many of whom were associated with preparation of the Swiss Pharmacopœia Supplements themselves. The material of the book is divided into two parts, in the first new monographs, and in the second alterations and extensions. It is arranged alphabetically in the first part under the Latin titles of the Official Monographs, the majority of which are chemical. The layout of each section follows a standard pattern based on that of the official monographs to which they refer. Methods of preparation are described in some detail, followed by a description, with explanation, where appropriate, of properties, tests for identity and assay. Incompatibilities are explained, special formulations described, and special points relating to stability are discussed. The graph showing the relationship between vitamin C stability and pH is a particularly useful example of the type of information given under this heading. Still further sections provide useful background information to the official monographs on vaccines and sera; bacteriophage are also discussed. Comparatively few sections relate to crude drugs and galenicals, but these like the paragraphs on compressed tablets, lozenges, ointments, etc., have been treated equally thoroughly. Part 2 is concerned more with general processes, such as sterilisation, the use of preservatives, spectrophotometry, the measurement of hæmolytic activity, pyrogen tests, etc. The book contains numerous references to original literature, much of recent origin.

J. B. STENLAKE.

*METHODEN DER ORGANISCHEN CHEMIE* (Houben-Weyl). Fourth edition. Edited by Eugen Müller. Volume III. Part I. Physikalische Forschungsmethoden. Pp. xxx + 954 (including 448 illustrations and Index). Georg Thieme Verlag, Stuttgart, 1955. Moleskin: DM.162 00.

The present volume is one of two in this series devoted specifically to physical methods. Part II, Volume III, has already been published. The sixteen chapters of the present volume (Part I) cover thermodynamic methods; kinetic studies; measurements of density, solubility, vapour pressure, molecular weight (also macromolecules), surface tension, and surface activity; calorimetric methods; determination of molecular shape with the aid of space models; statistical assessment of error in physical methods; crystal geometry and the polarising microscope, crystal flow and anisotropy; mass spectrometry; measurement and use of radioactive and non-radioactive isotopes in organic chemistry. This volume, in line with the general emphasis on practical methods found throughout this series, is freely and excellently illustrated with sketches and

diagrams of specific pieces of apparatus. Throughout the book much useful information has been compressed by tabulating methods of experiment and physical constants, and also by the use of graphs. The book is excellently referenced, covering the literature up to 1955, though many of the more important methods are described in such detail as not to require further reference. The value of the information contained in this volume outweighs any disadvantage arising from a German text.

J. B. STENLAKE.

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(ABSTRACTS *continued from page 814.*)

was sodium 3:5-diacetamido-2:4:6-triiodobenzoate, sodium diatrizoate; acute intravenous LD50 values for the species mentioned were between 11·3 and 14 g./kg. With acutely lethal doses of the drug, death occurred between a few minutes and three hours as a result of massive pulmonary hæmorrhage and consequent right heart failure. Local tissue toxicity was very low as judged by the absence of injury to the tunica intima of the marginal ear vein of the rabbit with repeated injections of a 50 per cent. solution of the salt. In doses of 0·5 to 2·0 g./kg., sodium diatrizoate had no consistent effects on blood pressure, heart rate or respiration in the cat or dog; ganglionic transmission through the cat superior cervical ganglion and the response to serial carotid occlusion in the dog were likewise unaffected. The drug was well tolerated by rats when given intravenously in five consecutive daily doses of 0·5 and 2·0 g./kg.; under the same conditions 4·0 g./kg. caused one death and renal tubular nephrosis in five out of nine rats. In monkeys, sodium diatrizoate was well tolerated at three successive doses of 0·5, 1·0 and 2·0 g./kg. No significant hæmatological or histological changes were observed with these doses. Clinical studies on the drug have demonstrated excellent visualisation of the urinary tract, with low incidence of minor side effects. G. P.

**Tryptophan and 5-Hydroxytryptamine in Patients with Malignant Carcinoid, Studies on.** S. Udenfriend, H. Weissbach and A. Sjoerdsma. (*Science*, 1956, **123**, 669.) Patients suffering from metastatic malignant carcinoid, a relatively rare disease, show symptoms of intestinal hypermotility, bronchospasm, vasomotor disturbances and cardiac lesions. Blood levels of 5-hydroxytryptamine (5-HT) in these patients were from 0·6 to 3·0 $\mu$g./ml. compared with 0·1 to 0·3 $\mu$g./ml. in normal subjects. Urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of 5-HT, was 70 to 800 mg./day in carcinoid patients compared with 2 to 9 mg./day in normal controls; this feature was diagnostic of the disease. After administration of 2-$^{14}$C DL-tryptophan to three of the patients the excretion of labelled 5-HIAA demonstrated that tryptophan is the precursor of 5-HT and its metabolites. With a daily intake of 500 mg. of tryptophan, as much as 60 per cent. was converted to 5-hydroxyindoles, whereas in normal subjects only 1 per cent. was metabolised in this way. Nitrogen balance was just maintained in the carcinoid patient with daily amounts of tryptophan three to four times those required for balance in normal subjects. The altered tryptophan metabolism in the carcinoid patients results in less of the amino-acid being available for normal body requirements, with subsequent weight loss and hypoproteinæmia; pellagra has also been reported in some cases. The disease symptomatology is probably related both to this tryptophan deficiency and to 5-HT excess. G. P.