# REVIEW ARTICLE

## STRUCTURE—ACTIVITY RELATIONSHIPS

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THE relationship between the chemical structure of a compound and its effect on a biological system is most frequently considered in rather a narrow sense, referring only to synthetic organic compounds or even to a limited class of compounds. Moreover, consideration is often restricted to a limited type of biological response, or to an ill-defined end response such as death of the organism. All naturally occurring compounds in living systems are possessed of biological "activity" of some kind, and even if their function is somewhat static as in structural components, they have at least been synthesised by dynamic biochemical processes. Even the simplest of compounds, water, is biologically active, as it participates not only as a solvent but as one of the most important reactants in the living cell. A full analysis of structure-activity relationships must therefore embrace the whole of classical biochemistry, to which must be added the growing mass of knowledge accumulating as the result of the development of chemotherapy, the fermentation industries, the plant protection industry and the control of organisms attacking manufactured goods. The temptation to narrow the field of enquiry is strong, because by doing so, it is possible to arrive at greater precision in correlating structure and activity. Moreover, it is often said, and with a considerable measure of truth, that fundamental knowledge is so fragmentary that any attempt at broad generalisations is premature. This negative attitude should be resisted, for hypotheses are valuable in crystallising thought and suggesting experiments. There are, in fact, certain valuable basic data, or at least practical working rules, which can act as guides, and a broad enquiry can at least help to define those areas where the gaps in knowledge are greatest and upon which research should be concentrated.

Living organisms, from the point of view of this essay, are considered as dynamic physico-chemical systems. They are dynamic, not necessarily in the sense of movement, but because the living process is one of rapid and orderly chemical change, from the intake of nutrient to the output of waste products. The structure of every molecule must be viewed in relation to its environment, and to its metabolic precursors and successors. Living organisms vary greatly in their biochemical complexity, and to a large extent the study of structure-activity relationships is dependent on the possibility of biochemical dismemberment of the organism, so that the working of the component parts may be examined in isolation. The metabolic processes of micro-organisms, for example, are susceptible to experimental study not only by correlation of the effect of nutrients and foreign compounds on the metabolism, but also by the isolation and

study of individual enzymes and their substrates. Again, it is possible by isolating a particular organ from an animal, for example, thyroid gland, liver, or nerve, to study the effect of chemical compounds on the functioning of the organ in question. Natural associations of enzymes may be studied in isolation from the whole organism by the use of particles such as mitochondria or chloroplasts. This kind of approach has been of immense value, and it will continue to provide basic data of crucial importance. Nevertheless, it has its limitations, and erroneous conclusions may be made because the behaviour of a biochemical system in isolation may not be the same as when it is fully integrated with the interdependent system of the whole organism. Questions such as cell permeability and the inductive formation of intracellular enzymes may have a decisive influence in the natural condition, but may be largely ignored in some of the classical types of biochemical experiment. In the higher organisms, the controlling influence of the nervous and endocrine systems, each with its own complex biochemical characteristics, plays an important part in determining the response of the whole organism to a particular chemical compound. The ability to express psychosomatic medicine in chemical terms is still far distant, though courageous speculations are beginning to appear in certain directions. A special word of caution is needed when "biological activity" means death. A bactericide kills the organism but structure can hardly be correlated with this kind of activity except in restricted series of chemical compounds. It may be legitimate, for example, to correlate structure and bactericidal activity amongst phenols, quaternary ammonium compounds, sulphonamides, or penicillins. It is useless to search for a significant common structural feature in the four classes of compounds named, because they have none. What matters is the biochemical mechanism of the process which results in death of the bacteria.

In a complex living organism, there are many factors which determine whether a foreign compound can exhibit a particular type of biological activity. First and foremost, the compound must be able to reach, in adequate concentration, the site at which the desired biochemical action can result. This may be a particular organ or a parasite. In its transport to the site of action, the compound must overcome a number of barriers. These barriers are partly physical and partly chemical. Physical factors, all affected by the chemical constitution, include quantitative aspects of solubility in aqueous and lipoid phases, membrane permeability, and capacity for adsorption on macromolecular species in solid or solution form. All these affect the rates of absorption and excretion of the compound in question, and hence the concentration at any particular place in the organism. The principal chemical factor determining access to the site of biochemical action is the reactivity of the compound. If the compound is highly reactive, it is likely to be wasted by chemical interaction of a non-specific nature with various chemical components of the organism, even with water itself. If it contains groupings which are susceptible to enzymatic attack, it may undergo chemical change. This change is generally known as detoxification because it frequently

results in the formation of a derivative, for example, glycuronide or other conjugate, which is rapidly excreted, and which has lost the characteristic biological activity of the parent compound. Metabolic change of this kind can, however, actually increase the biological activity, and examples will be quoted later. The second necessary property of a compound which is expected to be biologically active in a complex organism hardly requires statement. It is the intrinsic ability to participate, preferably in a specific way, in the functioning of the particular biochemical system concerned. Thus in the biological activity of a foreign compound, structure-activity relationships are governed by a large number of competing factors. Certain structural features will favour intrinsic activity, and certain features will favour the access of the compound to the desired site. Only when these features are favourable in both respects will activity result. Any one structural feature may thus limit the overall effect.

## Activity Through Physical Effects

There are certain compounds (or even elements) which have little or no chemical reactivity but which nevertheless can exhibit biological activity, perhaps by a rather non-specific damping down of vital biochemical reactions in the living organism. Such activity is usually reversed on removal of the drug and is often described by a broad use of the term narcosis. Narcotics, in this sense, owe their activity to physical effects and there is a close correlation between biological activity and certain particular physical properties. In so far as these physical properties can be correlated with structure, the structure can be correlated with biological activity. It is found, however, that elements and compounds of a widely different chemical nature exhibit the relevant physical property in like degree; correlation of structure and activity in these cases would therefore probably require resolution at the atomic level.

There have been various theories or empirical rules for correlating activity and physical properties. There is the Overton-Mayer concept whereby narcotic activity was related to the lipid: water partition ratio of the compound, and the Traube hypothesis relating narcotic activity to the effect on the surface tension of water. Each of these has served its usefulness, and they were for a time considered as rival theories. Considerable clarification resulted from an analysis of the situation made by Ferguson in 1939[1]. He recognised that in homologous series of compounds, the variation in toxic concentrations exhibited the same type of relationship as variation in physical properties. Thus, straight lines were obtained by plotting the number of carbon atoms on the chain of normal primary alcohols against the logarithm of either the toxic concentration against *B. typhosus* or the solubility, vapour pressure, surface-tension lowering capacity or partition coefficient betwen water and cottonseed oil. Ferguson emphasised that the concentration required to produce a given biological activity was usually expressed as the concentration in the surrounding medium, but that what is really required is to express the concentration at the site of action, the "biophase". Assuming an

equilibrium state between the various phases in the organism, the chemical potential ("activity" in the thermodynamic sense) will be the same in all phases. All that is necessary, therefore, is to calculate the thermodynamic "activity" in the surrounding medium, which can be done from measurements of vapour pressure or, approximately, from solubilities. In this way, it was shown that ascent of an homologous series revealed a gradual increase of the "activity" towards unity, at which point there was a cut-off, higher members having reduced biological potency. This, of course, is a familiar experimental finding, well exemplified by the fact that the insecticidal activity of normal thiocyanates reaches a maximum at about $C_{10}$ and the bacteriostatic action of *p*-alkylphenols at $C_5$.

Physical toxicity of the kind described may be exhibited by elements and compounds of widely differing structures. Even though the external concentrations required to produce a particular biological response may vary greatly, the chemical potential lies within a fairly narrow range which is a characteristic of the biological system in question. Illustrations have been provided by Ferguson[1] and by Gavaudin[2]. One particularly striking example compares the mitotic activity on plant cells of nitrous oxide, nitrogen, argon and propane[3].

## Effect of Physical Properties on Chemical Toxicity

From the phenomenon of physical toxicity, as outlined above, it follows that the biological activity of a compound which acts chemically will be greatly influenced by its physical properties. This is a familiar experience in chemotherapeutic research, where substituent groups in any particular chemical type can influence both the intrinsic activity and the ability to penetrate to the site of action. The homologous series effect is not confined to purely physical toxicity, but has frequently shown up in compounds where the intrinsic activity is undoubtedly associated with the occurrence of a chemical reaction *in vivo*. The same consideration applies for example to the effect of halogen and alkyl substituents in an aromatic or heterocyclic ring. Many examples have been quoted by the author[4].

Apart from the effects of structure on such physical properties as solubility and volatility, one of the most important of physical properties is that of molecular shape. Most biochemical reactions in the living organism are mediated by the intervention of macromolecular catalysts, the enzymes. All enzymes so far discovered are proteins, and their characteristic specificity is undoubtedly the consequence of an ordered three dimensional arrangement of the component parts, permitting the access of the natural substrates (and certain structurally related molecules) to the site where they are activated. The shape of the substrate molecule, which may be relatively small, is thus of vital importance. One has only to mention the differences in biological activity between stereoisomers to illustrate this.

A different kind of example of the effect of substituents on the shape of the molecule is provided by certain substituted *p*-aminobenzoic acids. Substitution of a halogen atom at the position *ortho* to the carboxylic group provides derivatives can can function biologically as analogues of

*p*-aminobenzoic acid, but there is a progressive lowering of the potency as the size of the halogen atom is increased from fluorine through chlorine to bromine. Here, one may envisage the largest halogen atom as providing the greatest impediment to the approach of the molecule to the enzyme "surface". Similar reasoning could explain the inactivation of synthetic auxins of the phenoxyacetic acid class by the introduction of two *ortho* substituents. In other words, steric hindrance is as significant in biochemical structure-activity relationships as it is in preparative organic

TABLE I

SOME DRUGS AFFECTING THE NERVOUS SYSTEM

| Formula | Type of activity |
|---|---|
| $Ph_2CH{\cdot}OCH_2CH_2NMe_2$ | Antihistamine |
| $Ph_2C\langle$ ($CH_2CHMeNMe_2$, $CO{\cdot}Et$) | Analgesic |
| $Ph_2C\langle$ ($CO$—$NH$, $NH$—$CO$) | Anticonvulsant |
| $(Cl{\cdot}C_6H_4)_2CH{\cdot}CCl_3$ | Insecticide |
| $Ph_2C\langle$ (piperidyl NH, $OH$) | Ataraxic |
| $Ph_2C\langle$ ($COOCH_2CH_2NEt_2$, $OH$) | Anti-acetylcholine |

chemistry. Other examples have been provided by the work of Landsteiner[5] and Pauling[6] on the serological reactions of artificial antigens prepared by attaching foreign molecules, notably substituted benzene derivatives, to proteins. The immune sera to such artificial antigens exhibit cross-reactions with proteins derived from determinant groups of related shape.

One of the best known examples of the deliberate use of the concept of molecular shape in synthesising biologically active molecules is the oestrogenic compound, stilboestrol. Here, the characteristic biological activity of the natural oestrogens was achieved by making a molecule in which two hydroxyl groups were separated by a structure giving the same spatial characteristics as in the natural steroids. Reproduction in a similar way of the biological activity of important natural products has been achieved in several other well-known cases. Examples are to be found amongst vitamins, in synthetic morphine-like analgesics and in the substituted phenyl- and phenoxyacetic acid auxins, which behave like indolylacetic acid. More recently, it has become apparent that a diphenylmethyl substituent, attached to various functional groups, confers properties which favour activity in the nervous system. The reason for

this is not clear, but it may well be a question of access to the site of action. Examples are given in Table I.

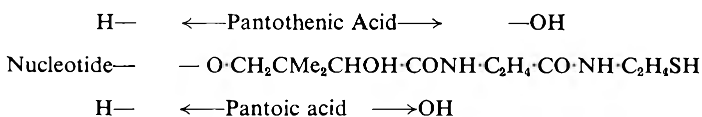
## Activity Through Chemical Reactions

The biological activity of many of the normal chemical constituents of a living organism is due to their ability to enter into a chemical reaction. They may, in addition, use forces of a physical nature. This is particularly, and perhaps predominantly, the case with macromolecules, where interaction is due to a multiplicity of points of loose attachment in stereochemical juxtaposition. Thus the binding of an apoenzyme to its substrate may depend on the forces of electrostatic attraction, hydrogen bonds or van der Waals forces. The same is probably true of antigen-antibody interactions. Many of the simpler chemical reagents (as distinct from catalysts) of the living organism, however, are the smaller molecules, the metabolites and the coenzymes. These compounds enter into well-defined chemical reactions, and the same is true of many biologically active foreign compounds. One of the simplest ways in which a foreign compound may affect a living organism is by entering into an irreversible chemical reaction with a vital metabolite. A good example is the reaction of vital thiol compounds (enzymes or smaller molecules) with heavy metals such as copper or mercury, or with a reagent like iodoacetic acid. Biological activity in such cases is usually non-specific, and manifested as a general toxic effect. The foreign compound is highly reactive, and with highly reactive compounds the possibilities of finding biological activity of a specific kind are remote. Many biologically active compounds have the character of acid anhydrides. Highly reactive anhydrides will enter into non-specific reactions with the first chemical constituents of the organism which they encounter, even with water. They therefore have no chance to exhibit specific effects. With a lower degree of reactivity (but not too low) they may resist attack by water and certain other agents, and so be able to penetrate to a point in the organism where there is an appropriate natural chemical with which they may react. Examples of such anhydride-like compounds are A.T.P., acetylcoenzyme A, penicillin, and the organophosphorus insecticides. Chemical reactivity, therefore, must be delicately poised.

When considering structure-activity relationships in compounds foreign to an organism, it is necessary to discuss the phenomenon of competition between metabolites. Briefly stated, this amounts to the simple conception that the place of a normal metabolite in a biochemical sequence may be taken by a foreign compound of related structure and electrochemical properties, with disruption to varying extents of the dynamic function. The metabolite competition hypothesis has resulted in considerable theoretical advances during the last two decades. The empirical discovery of antibacterial agents has led to intensified research on the biochemistry of micro-organisms, particularly since the discovery of the metabolic importance of *p*-aminobenzoic acid in 1940. Even if it is accepted that in this field theoretical explanation has hitherto generally followed after the empirical discovery of activity, the hypothesis has a

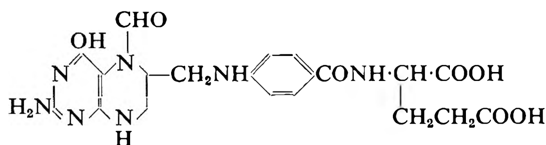
predictive value which will become more important as basic biochemical knowledge increases. Although Quastel and Wooldridge had clearly demonstrated competitive antagonism in an enzyme system as far back as 1928[8], the antibacterial action of sulphanilamide was not predictable in 1935, because at that date the biochemical significance of *p*-aminobenzoic acid was not known. Competitive antagonists to various essential metabolites and coenzymes have since been successfully synthesised, and there are many examples in the cases of nicotinamide, pantothenic acid, thiamine, biotin and various amino acids, purines and pyrimidines[4].

If, in a biochemical reaction sequence, it is desired to synthesise a competitive antagonist to a particular component, there are certain considerations which can be applied. There should be a structural similarity to the metabolite, so that the antagonist can occupy the site on any enzyme "surface" which is normally occupied by the metabolite. Groups which are responsible for the enzyme-substrate binding must, therefore, be retained, but groups which participate in the reaction sequence may be modified. Thus sulphanilamide resembles *p*-aminobenzoic acid in its shape, in the aminophenyl residue and in the fact that they are acids of comparable strength. The antagonist differs from the metabolite in that the natural carboxylic group, which in the sequence must react with glutamic acid, is replaced by a grouping which can undergo no such reaction. Similar considerations undoubtedly apply to antagonists of thiamine, biotin and riboflavine, but space does not permit of more detailed illustration. A more complex example may be cited in relation to pantothenic acid, illustrated as part of the formula of coenzyme A, below.




Antagonists to pantothenic acid result by substitution its COOH group with $SO_3H$, SH, $-CO\langle\bigcirc\rangle Me$, $-SO_2\langle\bigcirc\rangle Me$ or $-CONHNH_2$, all of which groupings are incapable of reacting with mercaptoethylamine. Replacement of the terminal OH group responsible for combining with the nucleotide by hydrogen produces an antagonist under defined experimental conditions. An antagonist of pantoic acid was also produced by replacing the terminal OH by H. The simultaneous replacement in pantothenic acid of the terminal OH by H and of the COOH by $SO_2NH_2$ gave a compound which was biologically inert[9,10]. This may well be because of the inability of the synthetic compound either to bind to an enzyme or to react with either of the adjacent compounds in the biochemical sequence. In other words, its structure is such that it is prevented from any type of participation in the specific chemical reactions concerned.

Another interesting class of metabolite antagonists has been derived from folic acid. This is depicted in the catalytically functional form of folinic acid (I), the *N*-formyl derivative of tetrahydro-pteroylglutamic acid.

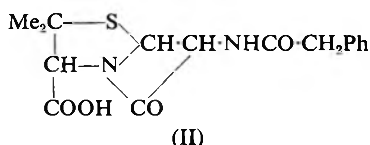


(I)

In this molecule, the *N*-formyl group is believed to provide the carbon atom which mediates various important "one-carbon" biosyntheses. Examples are the formation of purine rings by cyclic formylation of an open-chain intermediate, conversion of glycine to serine, uracil to thymine and homocysteine to methionine. Although exact details of the biochemical reactions are not yet known, it has been observed that various derivatives will function as folic acid antagonists, and their activity is now readily understood from their structures. One is pteroylaspartic acid, which obviously upsets the function of the glutamic acid moiety of the natural compound[11]. In aminopterin, the pyrimidine OH is replaced by $NH_2$, thus providing an opportunity for "fixation" of the formyl group by ring closure[12]. In methopterin, the linking $-CH_2NH-$ group is *N*-methylated. There is evidence, at least from the natural occurrence of 10-formylpteroic acid (rhizopterin), that this N atom is concerned in the formation or function of folinic acid, to which purpose methylation would be inimical. In contrast to the case of pantothenic acid, simultaneous alteration at two vital points in the molecule has also produced antagonists. These are A-methopterin, which contains both the amino group of aminopterin and the methyl group of methopterin[12], and the (4) amino analogue of pteroylaspartic acid[14]. Obviously in spite of the double change of structure, both these compounds retain an affinity for the vital enzyme or enzymes concerned.

In the examples mentioned above, the competitive metabolites result in a major disruption of the total metabolism, resulting often in the death of the cell. The overall effect of introducing competitive metabolites, however, need not be so drastic. If *E. coli* is grown in a medium containing *p*-fluorophenylalanine, this synthetic amino acid is incorporated into proteins in the places normally occupied by the structurally related amino acids, phenylalanine and tyrosine. Some, but not all, of the resulting proteins retain their characteristic biological activity[15]. In a similar way, structural analogues of the normal purine and pyrimidine bases can become incorporated into nucleic acids. In fermentation of *Penicillium chrysogenum*, a variety of penicillins can be produced, and in the normal manufacture of penicillin G, it is the practice to add phenylacetic acid or a suitable derivative to the medium in order to provide the mould with an excess of one of the normal precursors. This results in the predominant production of penicillin G, with its phenacyl side chain (II). If phenoxyacetic acid or various substituted derivatives of this or of phenylacetic acid are employed "unnatural" penicillins are obtained, such as the important penicillin V (phenoxymethylpenicillin). In the same way, numerous biologically active analogues of vitamin $B_{12}$ have been produced experimentally, by addition to the culture medium of structural analogues of the

natural base, 5:6-dimethylbenziminazole. Examples of analogues which are effective in this way are benziminazole itself, and some halogen- or even nitro-substituted derivatives, 4-chlorobenztriazole, adenine and some substituted derivatives[16]. At a relatively simple chemical level the production of halogen-containing mould metabolites can be influenced by the quantity and nature of the inorganic halide in the culture solution. Examples are griseofulvin (III) and the tetracyclines, for example, aureomycin (IV).

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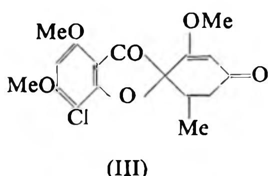


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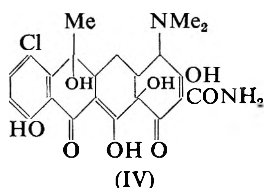
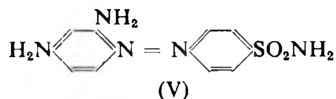



(IV)

## Drug Metabolism

When a foreign compound enters a living organism, there are two effects to be considered, the response of the organism and the chemical effect of the organism on the drug. The second of these is of great practical importance in chemotherapy, where one of the problems is to ensure an adequate concentration of the biologically active compound at the site of action. Drugs can undergo metabolic alteration to varying degrees. This results from interaction with the normal biochemical reagents of the cells, though there are special circumstances, discussed later, where the drug can alter the cell metabolism in a manner inimicable to its own molecular integrity. Such metabolic changes are commonly referred to as detoxification processes, because they frequently result in the excretion of the foreign compound. The term is unfortunate, however, because the chemical changes undergone by a drug may on occasion result in the production of a derivative which is more toxic to the organism (or a parasite) than the drug itself. Well known examples are the greatly increased trypanocidal activity of pentavalent arsenicals on reduction to the trivalent state in the animal body, the reduction of the azo dye, Prontosil Rubrum (V), to sulphanilamide, and the increased antimalarial activity of proguanil (VI) through its metabolic oxidation to a dihydrotriazine (VII)[17]. Both these examples relate to parasites, but there are also cases where parasites are not involved. The carcinogenic action of certain aromatic amines is probably indirect, the true active agents being oxidation products. The auxin-like activity of substituted $\omega$-phenoxyaliphatic acids $ArO(CH_2)_nCOOH$, where $n$ is an odd number, arises through their degradation by $\beta$-oxidation to the lowest homologue ($n = 1$) which is active in its own right[18]. The same applies to the

toxicity of ω-fluoro-aliphatic acids containing an even number of carbon atoms[19]. Similarly, the activity of esters of synthetic auxins probably follows their hydrolysis, though this has never been proved experimentally. The toxicity of methanol to various types of organism may well be due as much to its oxidation to formaldehyde as to the inherent toxicity of the alcohol.



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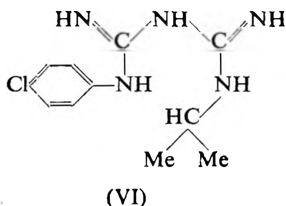

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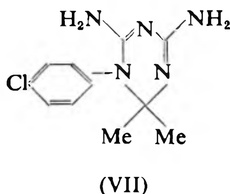


(VII)

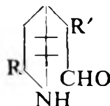
The commonly occurring metabolic reactions whereby a foreign compound may be changed chemically are (*a*) oxidation and reduction, (*b*) hydrolysis and condensation, (*c*) alkylation and dealkylation. Oxidation of aliphatic chains has already received brief mention in the previous paragraph, and it offers an explanation of a structure-activity relationship which is occasionally encountered, namely an alternation in potency between odd and even numbered members of an homologous series. Aliphatic or aralkyl alcohols and amines may be oxidised by animals through aldehydes to carboxylic acids. Hydroaromatic or reduced heterocyclic rings may be aromatised by oxidation. The hydroxylation of aromatic compounds to phenols (and their subsequent conjugation with solubilising groups) is a familiar "detoxification" process in animals exemplified in the case of aniline and the polycyclic carcinogenic hydrocarbons. Reductions in the animal body have already been mentioned in the case of pentavalent arsenic and a particular azo compound. They also occur with nitrocompounds. Other azo compounds which are split in the animal body are certain carcinogenic azo dyes. In yeast and green plants, aliphatic aldehydes, for example, chloral, are reduced to alcohols.

Condensation reactions in normal biochemical processes are represented in the biogenesis of many important macromolecules such as proteins, polysaccharides and nucleic acids, and in processes of esterification such as fat formation. Many of these reactions can be looked upon as acylation reactions, and acylation of foreign substances is frequently encountered. In the animal body, amines are frequently acetylated, as for example, in the case of aromatic amines such as the sulphonamide drugs and aniline itself. The actual process of acetylation is mediated through the intervention of acetyl-coenzyme A, which functions as a mixed anhydride. Foreign carboxylic acids will often combine in the body with amino acids, as in the well known excretion of benzoic in

the form of hippuric acid. In birds, ornithine takes the place of glycine. Phenylacetic acid may be eliminated by some mammals in the form of a conjugate with glutamine. In dogs, some benzoic acid is excreted after condensation with glucuronic acid. Phenols frequently form sulphuric esters ("ethereal sulphates") but the choice of the conjugating molecule is influenced by substituents in the benzene ring[20]. Condensation of alcohols and phenols with sugars, the formation of glycosides, is more frequently encountered in green plants, and the nature of the conjugating sugar varies both with the nature of the alcohol or phenol and with the plant species[21]. Hydrolyses of foreign compounds are encountered in the breakdown of barbiturates by fission of peptide links, and in the hydrolysis of simple penicillin esters (themselves not bactericidal) in certain animal species, though not in man, to the bactericidal free acid penicillin.

Alkylation processes are more frequently considered in relation to the mode of drug action than to so-called detoxification processes, though the ubiquity of transmethylation reactions involving choline and methionine suggests that methylation of foreign compounds ought to be of frequent occurrence. A few examples are quoted by Williams[24], notably the conversion of pyridine to methylpyridium hydroxide which has been known for seventy years, and the similar quaternisation of various pyridine derivatives, including quinoline. This process, apparently, has some species selectivity, and does not occur in rabbits. The conjugation of many halogeno compounds, for example, benzyl chloride and halogen-substituted benzene compounds, with *N*-acetylcysteine amounts to an alkylation of the thiol group rather than a condensation. Dealkylation of, for example, ethers or tertiary amines is perhaps properly regarded as a special case of oxidation. The alkyl group may come off in the form of aldehyde, as in the case of the removal of the first methyl group from the carcinogen, *p*-dimethylaminoazobenzene[22]. In the dealkylation of the side-chain tertiary amino group in chloroquine[23], a suggested intermediate is the *N*-oxide. One of the metabolic changes of cinchona alkaloids in man is hydroxylation in the quinuclidine ring, and the author has suggested[4] that this may be a special case of oxidative dealkylation on the lines of VIII–X.

(VIII)



(IX)

(X)

## Drug Resistance

The biological activity of many compounds is affected in a quantitative way of continued exposure to sub-active concentrations of the compound. This is of great practical importance in the chemotherapy of bacterial infections and in the control of insect pests. Under these conditions, the bacterial or insect population may acquire a resistance to the action of much higher concentrations of the toxicant. This overall statistical

result on a multiplying population is probably due to a combination of two types of phenomena, one genetic and one physiological. In the present context it is only profitable to discuss the latter, in which the toxicant induces biochemical changes which favour the survival or continued function of the cells in question. The possible physiological basis for drug resistance has recently been discussed in general terms by Davis[25], who questions the general validity of two hypotheses which at one time found considerable favour. One was that the blocking of a biochemical pathway by a drug forced the organism to develop an alternative route. The other was that the organism elaborated increased quantities of the metabolite which was antagonised by the drug. The increased synthesis of *p*-aminobenzoic acid under sulphonamide bacteriostasis had early been advanced as a possible cause of sulphonamide resistance, but Davis considers this to be of dubious quantitative significance. A doubled level of thiol compounds in arsenic-resistant ticks has recently been reported[26], but unless the level of a particularly significant thiol compound was masked in the modest overall thiol increase reported, this also is a questionable explanation of the acquisition of arsenic-resistance.

A profitable field of enquiry has been opened up in recent years by the growing knowledge of enzyme adaptation and of bacterial permeases. It is only possible here to give an extremely brief and over-simplified account of these phenomena. The reader is referred to several recent review articles for more extensive, and to some extent speculative accounts[15,27–30]. The bare facts are that the production of normal enzymes within a cell can be stimulated to excess by the presence of their substrates and certain structural analogues thereof, and that the penetration of certain polar compounds into cells is mediated by enzyme-like permeases in the cell wall. Permeases, like intracellular enzymes, are capable of being induced or inhibited. Adaptive enzyme formation has mostly been studied in relation to the formation of penicillinase by *B. cereus*, induced by minute amounts of penicillin, and the induced formation of β-galactosidase. The increased quantities of penicillinase in *B. cereus* cause the organism to be resistant because the penicillin is destroyed by the enzyme. The induction is specific to compounds (penicillins, cephalosporins) of closely related structure[31]. Adaptive enzyme (and permease) formation occurs in the absence of cell division, but is dependent on the active synthesis of protein, which in turn is linked to the synthesis of nucleic acid. It is possible for a particular foreign compound to affect adaptive formation of either a permease or an intracellular enzyme, but not necessarily both. "Induced" *E. coli* metabolises various structurally related galactosides, but exchange of the glycosidic oxygen for sulphur affords compounds which, though they can penetrate the cell wall, are not attacked by galactosidase. When glactosidase is induced in *E. coli* in a medium containing *p*-fluorophenylalanine, the normal amount of the enzyme is formed, but no additional permease.

The theoretical relevance of all this to the problem of drug resistance is obvious, but examples of its application to drugs are as yet very few in

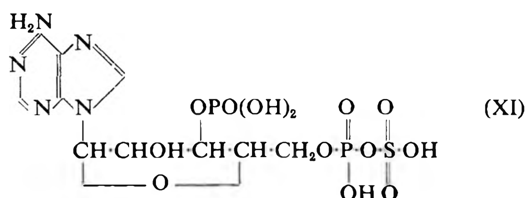
number. Penicillin resistance has already been mentioned. An interesting case has been provided by certain carcinogenic hydrocarbons. Administration of methylcholanthrene or of 3:4-benzpyrene to rats induces the formation in the liver of increased quantities of enzymes which hydroxylate the hydrocarbon and which demethylate carcinogenic methylated aminoazo compounds. The induction is prevented by ethionine, which is countered by methionine[32]. Insects rendered resistant to the insecticide, DDT, contain increased amounts of an enzyme "DDT-dehydrochlorinase" which converts it to an inactive compound. Some structural analogues of DDT which exhibit a synergistic effect with DDT against resistant insects appear to do so by inhibiting the enzyme[33]. The key position of nucleic acid in the phenomenon of drug resistance is emphasised in a recent brief report. If DNA, extracted from *Dip. pneumoniae* cells rendered resistant by exposure to A-methopterin, is added to normal susceptible cultures, these cultures acquire resistance to the drug[34]. It has been observed that azaguanine will inhibit the formation of adaptive enzymes in *Staph. aureus*. Guanine reverses this, not by preventing incorporation of azaguanine into RNA, but by allowing an increased production of RNA[35].

## Biological Action of Specific Structures

We have seen how, on general grounds, activity may be the result of a purely physical process or it may result from chemical reactivity within the cell, as regulated by the structure of the molecule. The concept of toxophoric groupings, by analogy with chromophoric groups, has only limited validity. Whereas all compounds containing chromophores are coloured, a particular grouping may confer biological activity only in some restricted compounds or classes of compounds. There are, nevertheless, certain fairly well-defined chemical reactions whereby foreign compounds can interfere in biochemical reactions, and it is of interest to illustrate by a few examples.

*Alkylating agents.* The alkylating ability of simple alkyl halides or other alkyl esters in preparative organic chemistry is a type of chemical reactivity which can be measured. In the biological activity of these compounds, the mechanism is chemical if they are sufficiently reactive or physical if they are not reactive. This was demonstrated by Ferguson and Pirie[36], who showed that alkyl chlorides above methyl, or bromides above propyl affected grain weevils by a physical process. Methyl chloride and the lower bromides, being more reactive, showed chemical toxicity. In rats, the ethyl ester of methane sulphonic acid will ethylate the thiol group of cysteine[37]. In more complex compounds, an alkylating function has in recent years been recognised as probably underlying the radiometic activity of nitrogen mustards, epoxides and ethylenimines[38]. These compounds react readily with anions, and the suggested site of chemical reaction in their biological activity is the (ionised) phosphoric acid residue in nucleic acid[39]. Alkylation is probably a cell reaction associated with the insecticidal activity of chlorinated hydrocarbons such as $\gamma$-benzene hexachloride. In addition to alkylation as a chemical

reaction of biological significance, one must consider the significance of alkyl groups as substituents in molecules which are active through some other chemical or physical process. The nature of the alkyl group will influence biological activity through its effect on physical properties and on reactivity. Classical examples are the alkyl substituted phenolic antiseptics and the alkyl thiocyanate insecticides. The effect of methyl substituents on the carcinogenic activity of certain polycyclic hydrocarbons is probably a chemically activating influence. In other circumstances, a methyl group may, for steric reasons, interfere with chemical reactivity in such a way as to destroy a characteristic biochemical reaction. Examples have already been given in a modified pantothenic acid and in the folic acid antagonist, methopterin. Another case is provided in the destruction of vitamin activity in nicotinamide by insertion of a methyl substituent in the 2-position.




*Acylating agents.* Several important natural products have been recognised through their function as acylating agents. They are mixed anhydrides, typified by adenosine triphosphate, "active sulphate" (XI), "activated" amino acids and *S*-acetylcoenzyme A. Foreign compounds which have an anhydride-like reactivity may be exemplified by penicillin and by the modern organophosphorus insecticides. Although the mode of action of penicillin in terms of organic chemistry is still obscure, it may well be associated with an acylating action. The case of the organophosphorus compounds is more clear. Here, various esterases (choline esterase, chymotrypsin, etc.), are acylated (possibly at the $CH_2OH$ of serine) with formation of a phosphorus-containing enzyme in which biochemical function has been destroyed. This is particularly important for choline esterase, which is intimately concerned with nervous transmission. The reaction is illustrated for di*iso*propyl phosphorofluoridate and an enzyme, EH.

$$F{\cdot}PO(OPr)_2 + EH \quad E{\cdot}PO(OPr)_2 + HF$$

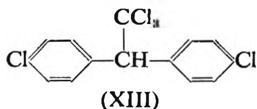
*Thiol reactors.* Thiol groups play an important role in natural processes because of their capacity for reversible oxidation to disulphides, their acylation to give so-called high energy bonds, and other characteristic reactions. Thiol groups are essential components of many apoenzymes, and they are of vital functional significance in smaller molecules such as those of coenzyme A and glutathione. It follows that compounds which can react with thiols may be expected, subject to the limitations imposed by their physical properties, to exhibit biological activity. The simplest examples are ions of heavy metals such as copper and mercury, and certain organometallics such as alkylmercuri salts and organic arsenicals.

Interaction of arsenicals containing trivalent arsenic with thiols is now the accepted mode of action of these compounds; the dithiol, dimercapto-propanol, acts as an antidote to arsenical poisoning by its preferential (cyclic) attachment to arsenic.

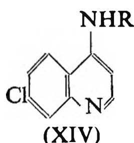
Perhaps the most important purely organic types of compounds which can react with thiols under physiological conditions are those containing the α-β-unsaturated ketonic structure, including open chain compounds and cyclic ones represented by unsaturated lactones and quinones. The cyclic structures are very commonly found in biologically active natural products which exhibit antibacterial, or sometimes herbicidal, activity. Studies of the reactivity of these, and of synthetic analogues, with thiols such as cysteine have been recorded[43,44]. The antibacterial activity of synthetic quinones has also been studied extensively from this viewpoint. Although more than one mode of action is feasible, there is considerable evidence that the activity of quinones, against Gram-negative organisms at least, is associated with their ability to react with thiol groups[45].

*Halogeno compounds.* The biological effects of introducing halogen atoms into organic compounds may be divided into physical and chemical effects. This has already been illustrated in the simple case of alkyl halides. The effect of halogen substitution in modifying physical toxicity has recently been made use of in the development of the anaesthetic, halothane (XII)[46]. Other physical effects are exhibited by halogen substitution in aromatic structures. Halogen-substitution in *p*-amino-benzoic acid is an example which has already been quoted. Halogenation of phenols and of quinones raises their bacteriostatic activity; halogenation in the appropriate positions of phenoxyacetic acid raises the herbicidal activity. In many cases, however, it is not possible to apportion the effect of halogen substitution clearly to physical effects or to effects on chemical reactivity. There is overlap, because the introduction of a halogen atom, whilst undoubtedly influencing physical properties, may also effect reactivity. A case in point is the insecticide, DDT (XIII). The biochemical function of the $-CCl_3$ group is probably through its reactivity. Without the two *p*-chlorine atoms the molecule is biologically inactive, but these chlorine atoms may be replaced by methoxyl without loss of activity. It is far from clear whether the *p*-substitution is influencing insecticidal action through an effect on physical properties, or through modifying the reactivity of the $-CH(CCl_3)-$ group, or through both. The same consideration applies to chlorine substitution in quinoline (XIV), acridine (XV) and biguanide antimalarials, in antibiotics such as aureomycin and griseofulvin, and in other chlorinated hydrocarbon insecticides. A clear case of biological activity arising directly from the introduction of a halogen atom into a position where it exhibits chemical reactivity is mustard gas and its nitrogen analogues, β-chloroethylamine derivatives, which are thereby converted into alkylating agents.

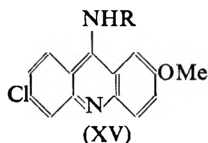
(XII)




(XIII)



(XIV)

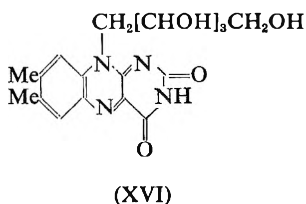


(XV)

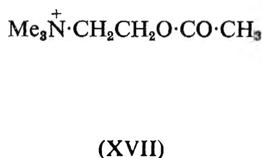
when R = $- \text{CHMe} [\text{CH}_2]_3 \text{NEt}_2$; XIV = chloroquine and XV = mepacrine

The substitution of a halogen atom (non-reactive) for hydrogen or methyl in certain natural metabolites frequently results in the production of competitive antagonists. Thus, fluoroacetic acid is metabolised to fluorocitric acid, an antagonist of citric acid[47]. 6-Fluoronicotinic acid competes with nicotinic acid. Substitution of chlorine atoms for the methyl groups of riboflavine (XVI) produces a riboflavine antagonist. 2-Methylnaphthoquinone has vitamin K activity; 2-chloronaphthoquinone antagonises this.

*Combination of two "active" groups in the same molecule.* Most workers who have attempted to synthesise biologically active compounds have at some time yielded to the temptation to introduce two "active" groupings into the same molecule. To take a hypothetical example, the untutored might attempt to obtain an improved bactericide by introducing a phenolic group into sulphanilamide, or to combine antimalarial with antibacterial activity by introducing a hydroxy group into the 8-position of the quinoline ring in chloroquine. There is a long history of such attempts, generally unpublished because of the disappointing character of the results. What happens is due to the fact that the antimalarial activity of chloroquine, for example, is intimately associated with the molecular architectural details. These would be greatly affected by the introduction of an 8-hydroxy group, so that although antibacterial activity might emerge in the hypothetical hydroxylated compound, the antimalarial activity would be expected to fall, probably to vanishing point.



(XVI)



(XVII)

Biological activity is, nevertheless, often exhibited by compounds with two or more "active" groups. In such cases the activity is usually of a highly specific kind, in terms of biological effect, and limited to compounds of closely related structure. To take a particular example, acetylcholine (XVII) is hydrolysed by the enzyme cholinesterase. The substrate is attached to the enzyme at two points which have a space relationship determined by the enzyme structure. These two are the anionic site, where negative charge on the enzyme surface attracts the positive

charge on the quaternary nitrogen of acetylcholine; and the esteratic site, where an electron donating atom in the enzyme attacks the carboxylic carbon. Specific affinity for this enzyme is, therefore, confined to compounds which contain two groupings, appropriately spaced and having similar electrochemical properties to the natural substrate. Compounds which do not have this structural relationship may yet attack cholinesterase (for example, the organophosphorus insecticides), but this attack is not specific to cholinesterase. Other esterases or proteolytic enzymes are also attacked by the organophosphorus compounds. This high specificity of action, based upon two- or multi-point attachment of the active molecular to a macromolecular catalyst finds its highest expression in the phenomena of virus multiplication, cell-division and immunity reactions. For accounts of this complicated topic the reader is referred to Landsteiner[5], to Sevag[48], and to various papers by Pauling and his associates published from 1940 onwards[6].

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# RESEARCH PAPERS

## THE MEASUREMENT OF OXYGEN CONSUMPTION IN SMALL ANIMALS*

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An apparatus is described which can be employed for the measurement of the oxygen consumption of one to six small animals in the weight range 25–500 g. The normal oxygen consumption of mice, rats and guinea pigs at 25° has been determined. The values obtained agree adequately with those of others. Evidence is given to support a claim that the guinea pig is, in some ways, a more suitable animal for the assay of metabolic stimulants than the rat or the mouse. Preliminary experiments have been made on the stimulant action of dinitrophenol and thyroxine on the oxygen consumption of guinea pigs.

During investigations of the toxicities of the dinitrophenols the need arose to measure their effects on the oxygen consumption of small animals.

A characteristic feature of the stimulant action of 2:4-dinitrophenol on laboratory animals is that it reaches and loses its maximum effect within the short time of 3–4 hours. In this it differs from thyroxine which shows a slower falling off of activity. Thus the apparatus used for assaying any metabolic stimulant must satisfy three basic requirements. First, it must be comfortable enough for an animal to settle down quickly to a resting or "basal" condition. Secondly, it must be sensitive to small changes in the oxygen consumption, and thirdly, its design should permit individual and group measurements to be made on several animals under nearly identical conditions[2,3].

The main objects of this communication are to describe an apparatus that has been designed to meet these requirements, to record its use in the measurement of the normal oxygen consumption of mice, rats and guinea pigs, and to report preliminary investigations on the measurement of the stimulating effects of 2:4-dinitrophenol and L-thyroxine on the oxygen consumption of the guinea pig.

### Construction of the Apparatus (Figs. 1 and 2)

A tank, approximately 3 ft. × 1 ft. × 1 ft. is constructed of Perspex sheet $\frac{3}{8}$ in. thick. Six Perspex cylinders are set horizontally between two long sides, with the ends just short of the outer faces. These cylinders form the outer walls of the six metabolism chambers (1–6) and are covered by circular Perspex plates 5 in. in diameter. The plates are held in place by two brass 4 BA bolts embedded in the tank walls and two wing nuts. Gas tightness is ensured by means of Vaseline or preferably a silicone grease smeared on the tank walls and on the plates. Each plate

* A demonstration of the apparatus described in this communication was given at the Physiological Society meeting held December 14–15, 1956[1].

is fitted with a glass tap (A–F, A1–F1) held in place by a rubber sleeve. The taps are connected by pressure tubing to the outlets of the two brass manifold tubes (M, M1). Each bank of taps is firmly fixed to a rigid wood frame (WF). This permits all plates to be removed rapidly in one operation. One of the manifold tubes is fitted with a master tap (G). The other is connected to an 80 ml. floatmeter and recorder by way of a $CO_2$ trap (PT) and a water trap (RT). Each metabolism chamber contains a smaller Perspex cylinder (6 in. × 3 in.) with a long slit (4 in. × $\frac{3}{8}$ in.)

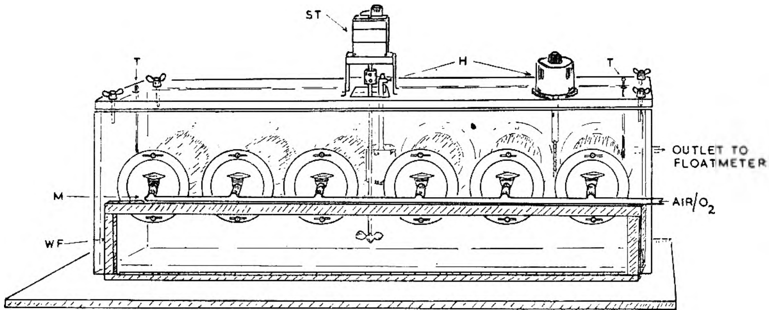


FIG. 1. Apparatus for the measurement of oxygen consumption. Floatmeter not shown. See text for description.

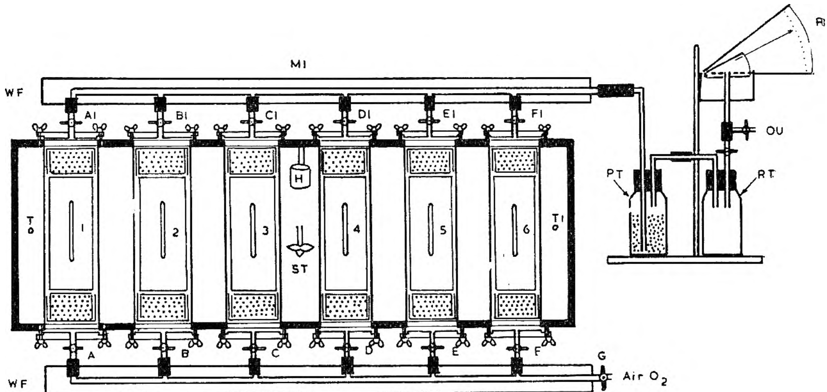


FIG. 2. Diagrammatic representation of the apparatus. Circular sealing plates are drawn just clear of side plates.

and fitted with four feet to keep a space between the two cylinders. A narrower (1 in.) cylinder is necessary for small rats and mice. In the spaces between the ends of the sealing plates and the inner cylinders are two "cartridges" containing Green 6–10 mesh Protosorb for trapping the $CO_2$. Each cartridge is $3\frac{3}{4}$ in. dia. × $1\frac{1}{2}$ in. deep and is constructed of perforated zinc in two close fitting halves. The cartridges fit snugly but not too tightly. The whole tank is filled with water. It is covered with a Perspex lid in which are fitted two thermometers (T, T), a stirrer (ST), and a thermostatically controlled heating device (H).

*Floatmeter Recorder*

The manifold Ml is attached by rubber pressure tubing to the $CO_2$ trap (PT). This is a small glass bottle (150 ml.) fitted with a two holed rubber bung and containing 20–30 g. Protosorb. Through one hole of the bung is a connection to the floatmeter trap (RT). Through the other is a large bore glass trap (OU) that can be opened to permit free current of air or oxygen through the apparatus without filling the floatmeter chamber. The floatmeter is fitted with a Perspex scale and the whole apparatus is calibrated in 10 ml. divisions. This is similar to that described by Tainter[4].

*Supply of Air or Oxygen*

Air is supplied by double bulb hand bellows, or oxygen through a bubbler from a cylinder.

While many methods require the use of oxygen or air enriched with oxygen[2,5,6] it was considered that air alone might be suitable for short time exposures. Clearly the total volume of air available would have to be liberal and the exposure time short for larger (300–500 g.) animals. In other words, it was essential to reduce the volume of oxygen consumed to a minimum. After some trials 10 ml. was adopted as a convenient volume. Consideration of the capacity of the apparatus indicates that each animal has available about 750 ml. of air. Therefore, if 10 ml. of oxygen is consumed the reduction of the total amount available is about 6 per cent. This can be replaced easily and rapidly by frequent aeration. No evidence has been obtained to suggest that this diminution of the total available oxygen is harmful to the animal.

## Method of Operation

The sealing plates on one side of the apparatus (A–F) are removed and one animal put into each chamber. The plates are replaced and the whole apparatus aerated thoroughly. Times are not taken until the animals have settled and the temperatures inside the metabolism chambers have reached 25°. Temperature equilibrium is established within 5 minutes although the settling time varies. As a rule it was found that rats and mice settled in 20–30 minutes[5] and guinea pigs in 5–10 minutes. During the settling period aeration is carried out frequently for the group and for the individuals.

*Group Determination on Six Animals*

After thorough aeration the apparatus is charged with sufficient air to bring the floatmeter arm to its maximum elevation. The master tap is closed. Timings by stop watch are taken for the consumption of three or four alternate 10 ml. volumes. The procedure is repeated until 12–16 values have been obtained that show less than 5 per cent variation.

*Single Animal Determinations*

The apparatus is aerated thoroughly and the floatmeter put into circuit as described previously. The master tap is closed. In order to measure the oxygen consumption of an animal in say chamber 1, Tap A is turned

off, taps B–F are turned on, and taps B1–F1 are turned off. The time taken for the consumption of a single 10 ml. volume is measured. Immediately after this the whole apparatus is thoroughly aerated and the procedure repeated for the remaining chambers. While individual measurements are made the remaining chambers are opened to the air.

*Temperature*

After some trials it was found that the most suitable environmental temperature was 25°. Lower temperatures (18–20°) appeared to cause restlessness in the animals and therefore delayed their settling time.

TABLE I

CORRELATION OF OXYGEN CONSUMPTION AND BODY WEIGHT OF GUINEA PIGS

| Sex | Regression coefficient | | | Equation |
|---|---|---|---|---|
| | D.F. | Value | S.E. | |
| Male .. | 46 | 0·625 | 0·034 | $y = 0{\cdot}625x - 0{\cdot}796$ |
| Female .. | 46 | 0·604 | 0·033 | $y = 0{\cdot}604x - 0{\cdot}738$ |
| Combined .. | 92 | 0·614 | 0·023 | $y = 0{\cdot}614x - 0{\cdot}765$ |

$y$ = log oxygen consumption (ml. $O_2$/min.) and
$x$ = log weight (g.)

Higher temperatures were also unsuitable because some of the compounds under investigation, for example, 2:4-dinitrophenol, 4:6-dinitro-*o*-cresol are strongly hyperthermic and their effects on metabolism are enhanced markedly by high environmental temperatures[7].

*Time Taken to Carry Out a Series of Determinations*

12–16 group and six individual determinations on six guinea pigs take about 0·3 hours depending on the weight of the animals. For six mice the same number of determinations takes about 1·5 hours.

*Treatment of Animals*

Animals are maintained on normal diets or as required. Food (but not water) is removed 1·5–2·0 hours before measurements are made.

## DETERMINATION OF OXYGEN CONSUMPTION

All results are calculated on a metabolism chamber temperature of 25°. No corrections have been made for atmospheric pressure.

*Normal Values*

Preliminary experiments with rats and mice demonstrated that their natural activity was high, and that it was difficult to get good resting values of oxygen consumption. Thus it was found that relatively mild exercise caused as much as a 50 per cent, and walking up to 150 per cent increase in the oxygen consumption of mice. Therefore, more attention was paid to the guinea pig. These are naturally quiet animals and have been employed by other workers for the assay of thyroid compounds[8]. Normal measurements were made on 96 guinea pigs (48 male and 48

female) in 16 groups of 6 animals per group. The weight range was 88–494 g. Twelve rats and twelve mice were also included in the study.

The normal oxygen consumption of guinea pigs measured over a wide weight range revealed a good relation between body weight and oxygen consumption. No significant difference in the response of the sexes was noted. Group and mean values are summarised statistically in Table I.

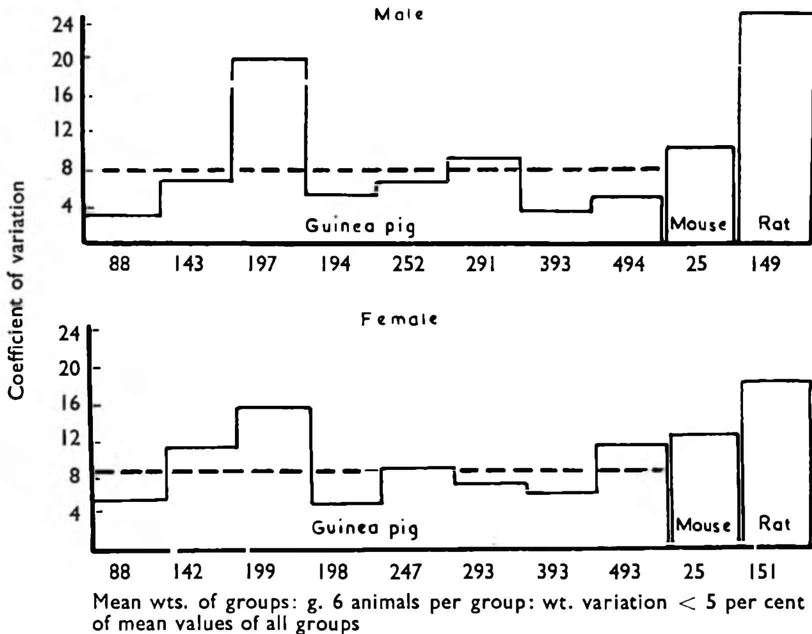


FIG. 3. Variation of oxygen consumption within weight groups of guinea pigs.

These results are in good agreement with those of Gaddum[9] and of Reineke and Turner[8]. In most weight groups there was reasonable uniformity in the oxygen consumption of the individuals. Only those animals in the weight range about 150–250 g. showed marked variation (Fig. 3).

Comparison of the mean of six individual determinations and the value of the group value revealed a fairly constant difference of about 15 per cent. No immediate explanation of this phenomenon can be offered but it may lie in the physical structure and operation of the system. From the point of view of assay methods the variation is not unreasonable provided that it remains constant, and preliminary studies on the assay of dinitrophenol suggest that such group measurements may be preferable.

## SERIAL DETERMINATION AND THE EFFECT OF EXCESS OXYGEN

Two experiments were made. In the first, air was supplied initially and the oxygen consumption of each of six guinea pigs was determined serially until six values per animal were obtained. The animals were removed, allowed to rest for two hours, and then replaced in the apparatus when they were supplied with air enriched with oxygen (air 1 pt.: oxygen

8 pts.), and six serial values obtained in a similar manner. In the second experiment twelve serial determinations were made on six guinea pigs before and after 4 daily injections of 120 $\mu$g. L-thyroxine sodium. Air only was supplied. The results are summarised in Table II and Figure 4.

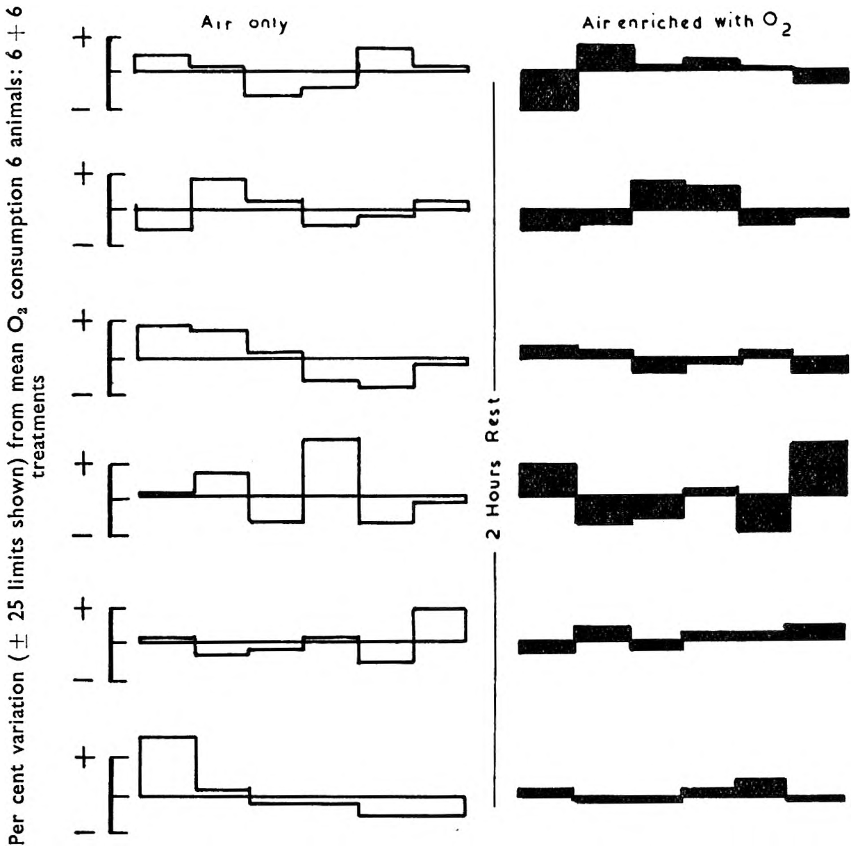


FIG. 4. Serial variation in the oxygen consumption of guinea pigs: air and then air heavily enriched with oxygen supplied.

The main result of this experiment is the demonstration that random fluctuations occur in the oxygen consumption of the guinea pig when serial measurements are made. In all probability this accounts for the fact that a series of group determinations made on six animals never shows much variation. In other words, it is likely that in any chronological series of observations random fluctuations of the individuals will cancel out to give homogeneous group values.

Variations greater than 20 per cent about the mean value of any one animal were rare and the average was 10 per cent. Only slight movements were noted at any time and the animals appeared almost motionless. By increasing the supply of oxygen there was an average increase in the consumption of about 20 per cent. Individual variations were less, but differences between animals were greater than when air alone was supplied.

## METABOLIC STIMULANTS

### 2:4-*Dinitrophenol*

In each of the two simple dose response experiments single doses of dinitrophenol were administered to guinea pigs by intraperitoneal injection of a 1 per cent aqueous solution (w/v) as its Na salt. The doses given were

TABLE II

SERIAL VARIATION IN OXYGEN CONSUMPTION OF SIX GUINEA PIGS (285–380 G.)

| Treatment | Oxygen consumption | | | |
|---|---|---|---|---|
| | ml. $O_2$/min. | | Coefficients of variation of the six mean values | |
| | Range of 6 mean serial values | Mean | Range | Mean (*a*) |
| Air .. .. | 4·19–5·71 | 4·72 | 7·1–19·0 | 14·7± 4·2 |
| Air–$O_2$ .. .. | 5·42–6·61 | 5·97 | 5·1–23·5 | 11·4±7·2 |

*Note.*—(*a*) If the S.D. of the mean coefficient of variation is accepted as a measure of scatter between the six animals mean serial values, then it is clear that air plus oxygen results in a more variable response than air alone in the ratio $\frac{7\cdot2}{11\cdot4} : \frac{4\cdot2}{14\cdot7}$ or 2·1 : 1·0.

5·0, 10·0, 20·0, 25·0, 30·0 and 35·0 mg./kg. Single determinations were made on each animal before and 1·25 hours after administration. Guinea pigs weighing about 300 g. were used. Dosing and determinations were carried out as follows:—

| Experiment | Total animals used | Dosing | | Determinations | |
|---|---|---|---|---|---|
| | | No. of levels | Animals/dose | Total | Type |
| 1. (Fig. 5) .. | 36 | 6 | 6 | 34 | Group and individual |
| 2.* .. .. | 18 | 6 | 3 | 18 | Individual |

* The response in the second experiment was similar to that in the first.

### *Thyroxine*

Two experiments were made. The first was a simple dose response, making single determinations. The second was to investigate the results obtained from many determinations.

Thyroxine was administered daily for four days to guinea pigs by the subcutaneous injection of L-thyroxine sodium dissolved in 0·1 per cent aqueous (w/v) sodium carbonate. Determinations and weighings were made on each animal before the first dose and after the last. Dosing and determinations were made as follows:—

| Experiment | Total animals used | Dosing | | Determinations | |
|---|---|---|---|---|---|
| | | μg. thyroxine/animal/day | Animals/dose | Before treatment | After treatment |
| 1. (Fig. 6) .. | 15 | 15, 30, 60, 120, 240 | 3 | 1 | 1 |
| 2. (Table III) | 6 | 120 | 1 | 12 | up to 12 |

Dinitrophenol administered intraperitoneally to guinea pigs caused an increase in the oxygen consumption which showed a general relation of log dose to log per cent increase in oxygen consumption per 100 g. body weight. Figure 5 illustrates this. On the whole, better results were obtained when 6 animals were used per dose, and close agreement was obtained between the group and mean individual determinations.

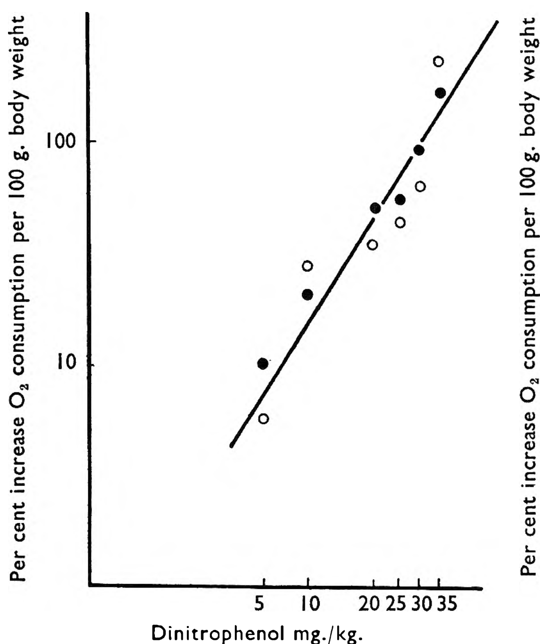


Fig. 5. Effect of dinitrophenol on oxygen consumption of guinea pigs. Single doses given by intraperitoneal injection. –●—●–group determinations on six animals per dose,–O—O–means of six individual determinations per dose. Line by observation.

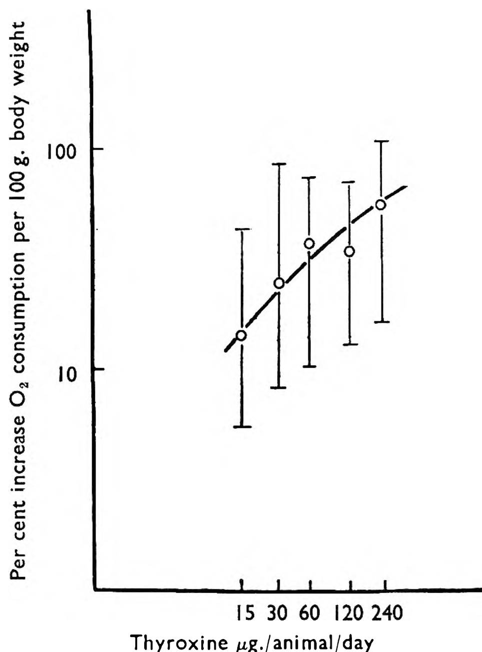


Fig. 6. Effect of thyroxine on the oxygen consumption of guinea pigs. Four animals per dose level. Mean and scatter shown.

Administration of graded doses of thyroxine revealed a similar relation between body weight and dose although there was a fairly wide scatter (Fig. 6).

In the second experiment (Table III) the administration of four daily doses of 120 µg. of thyroxine to each of six guinea pigs gave expected results, although three of the animals became very weak during treatment. These three died before twelve readings could be taken. Therefore, responses were calculated on the mean values derived from the first three as well as from all available determinations. The former gave more homogeneous results particularly for the animals that died when progressive respiratory failure resulted in a slow respiration rate.

## Discussion

"Basal metabolism or the basal metabolic rate is an expression of the body in complete mental and physical repose and in the post absorbtive

state."[10] As this condition is difficult if not impossible to obtain and to assess in small animals it is better to aim at achieving a less absolute value.

If very small numbers of animals are being used in an assay of a metabolic stimulant then more strict adherence to true metabolic conditions will be necessary. However, preliminary investigations on designed assays using larger numbers of animals and a wide range of dose levels suggest that it is more important to ensure, as far as possible, a reasonable degree of physical inactivity in the animals throughout. The design of apparatus described appears to favour this requirement.

TABLE III

THE OXYGEN CONSUMPTION OF GUINEA PIGS BEFORE AND AFTER FOUR DAILY DOSES OF THYROXINE (120 $\mu$G./ANIMAL/DAY)

| | Before treatment | | After treatment | | Difference (response) (per cent) (b) | | |
|---|---|---|---|---|---|---|---|
| | | Mean | | Mean | | Oxygen consumption | |
| Animal | wt. (g.) | ml. $O_2$/min. (a) | wt. (g.) | ml. $O_2$/min. (a) | wt. | On all values | On first three values |
| 1 | 305 | 4·39 (12) | 276 | 4·85 (10) | − 10·6 | + 25·2 | + 25·5 |
| 2 | 352 | 4·76 (12) | 313 | 5·97 (12) | − 11·1 | + 27·6 | + 26·4 |
| 3 | 500 | 5·91 (12) | 429 | 5·95 (5) | − 14·2 | + 16·0 | + 13·7 |
| 4 | 352 | 5·39 (12) | 323 | 4·27 (12) | − 5·3 | − 14·4 | + 22·5 |
| 5 | 410 | 6·37 (12) | 394 | 5·93 (3) | − 3·9 | − 2·0 | + 10·1 |
| 6 | 355 | 4·79 (12) | 317 | 5·97 (12) | − 10·6 | + 40·5 | + 25·5 |

*Notes:*

(*a*) The total number of values obtained are in brackets beside the mean value of the oxygen consumption.
(*b*) In calculating the increase in oxygen consumption allowance is made for the weight loss in each case.

Of the animals studied, guinea pigs have proved to be the most suitable for assays, as even stimulation by dinitrophenol caused little disturbance to their natural quietness.

In selecting the best weight, consideration must be given to the variations in the normal oxygen consumption of those animals within the weight range of 150–250 g. As a general rule, it is suggested that heavier animals (300–400 g.) should be used. It is interesting to note that Reineke and Turner[8] record few values of animals weighing 150–250 g. Therefore, it seems likely that they encountered similar variations and rejected these animals as unsuitable for thyroxine assays.

At this stage it is not proposed to deal with the best methods of assay involving oxygen consumption measurements, but with an apparatus of the type described the procedure for group determination must be made as an initial routine procedure. This allows the animals time to settle and permits the efficiency of the apparatus to be checked. In addition, if all the animals have received the same dose of metabolic stimulant then the procedure will give added information on the response of the group.

Work is now in hand to determine the minimum number of measurements that must be made on individual animals in an assay, and preliminary experiments suggest that three may be sufficient. Clearly one

determination per animal has several disadvantages, not the least being that it necessitates the use of a large number of animals.

The results of the experiments described in this communication lend weight to the arguments that many animals are necessary for the assay of metabolic stimulants[2]. Although much information exists on the assay of thyroxine and its analogues some of the methods are not altogether satisfactory and require improvement. Assay methods for dinitrophenol and related compounds, for example, 4:6-dinitro-*o*-cresol have not been satisfactorily worked out, and as a rule observations have been made on very small numbers of animals. In view of the risks to man and to animals exposed to this type of chemical it is essential that these omissions are rectified and these complementary problems are now being studied in more detail.

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# COLORIMETRIC ESTIMATION OF DIGITALIS GLYCOSIDES WITH 2:4-DINITRODIPHENYLSULPHONE

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The reaction is carried out as follows. The glycoside or aglycone, dissolved in 4 ml. of ethanol, is mixed with 5 ml. of a 0·075 per cent solution of 2:4-dinitrodiphenylsulphone in ethanol followed by 1 ml. of 0·15N sodium hydroxide. Colour density is measured in a 0·5 cm. cell at 20° at a wavelength of 6,000 Å against a blank prepared as the reaction mixture but omitting the glycoside or aglycone. Maximum extinction values of digitoxigenin and of gitoxigenin, boiled with 0·4N acid, are obtained 3 minutes after adding the sodium hydroxide to the reaction mixture. When digitoxin or digitoxigenin are examined without any pre-treatment with boiling acid, maximum colour is obtained 5·5 and 4·5 minutes respectively after addition of the alkali. Molar extinctions of digitoxin and of digitoxigenin are 23,200 and 24,400 respectively: after boiling with 0·4N acid the molar extinctions of digitoxigenin and gitoxigenin are 29,100 and 18,100 respectively. The values are much higher than those obtained with other reagents. A reduction in the ethanol concentration of the reaction mixture only exerts a slight influence on the maximum extinction.

Most of the colour reactions for digitalis glycosides are based on the aglycone part of the molecule, especially the butenolide side chain. A number of reagents such as sodium nitroprusside[1], sodium $\beta$-naphthoquinone-4-sulphonate[2], *m*-dinitrobenzene[3], 2:5-dinitrobenzoic acid[4] and trinitrobenzene[5] have been recommended as reagents, but picric acid[6] and 3:5-dinitrobenzoic acid[7] are most frequently used. All of these reactions are carried out in alkaline solution.

In the assay of digitalis leaves the isolated glycosides are always accompanied by a yellow pigment, digitoflavone. This substance reacts with sodium hydroxide to give a yellowish-brown colour and this may interfere with the quantitative estimation. Figure 1 shows the absorption curves of aqueous or diluted alcoholic extracts prepared from the same weights of one sample of digitalis leaf and treated with sodium hydroxide. It will be seen that by the addition of sodium hydroxide to the diluted tincture (curve II) the light absorption increases. This must be due to the digitoflavone. Curve I for diluted tincture is nearly identical with that given by Rowson[8]. Curve III demonstrates that water extracts more pigment from the leaf than does ethanol.

Generally only part of the digitoflavone present in the leaf remains in the reaction mixture during a colorimetric assay. However, it may be seen that if this component is not completely removed, too high extinction values may be obtained, especially when absorption values are measured at about 5,000 Å as in the picric acid reaction. A search was thus made to find a reagent which produces a colour with digitalis glycosides in small amounts and with a maximal light absorption at about 6,000 Å. *m*-Dinitrobenzene gives a blue colour with digitalis glycosides and was

used by Canbäck[9]. The disadvantage of the reagent is that maximum colour intensity is produced at the moment of addition of sodium hydroxide and the procedure proposed by Canbäck is too time consuming for serial work. Moreover the molar extinction for digitoxin is not very high, being 11,100.

Of a number of different reagents investigated 2:4-dinitrodiphenylsulphone gave satisfactory results. The synthetic product had a melting point of 157·4°–158·2°; its solubility in ethanol is not great. The corresponding sulphide and sulphoxide do not react with digitalis glycosides. The reaction was carried out using aldehyde-free ethanol prepared by the method of U.S.P. XIII. The glycoside was dissolved in 4 ml. of ethanol, 5 ml. of solution of 2:4-dinitrodiphenylsulphone in ethanol added followed by 1 ml. of aqueous sodium hydroxide solution. The colour density of the solution was determined in a 0·5 cm. cell at a wavelength of 6,000 Å against a blank of 4 ml. of ethanol, 5 ml. of reagent and 1 ml. of sodium hydroxide solution. The spectral band width was 45 Å and the measurements were made with a spectrophotometer (Bleeker, Holland).

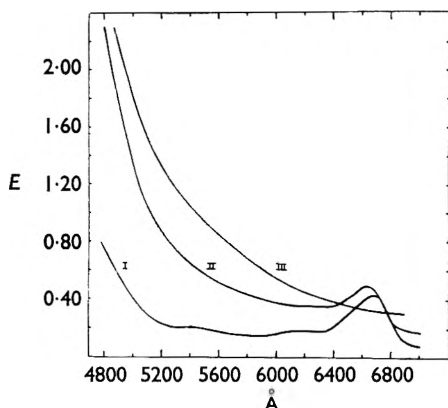


FIG. 1. Absorption curves of digitalis extractions with or without added sodium hydroxide. Curve I. Digitalis tincture (prepared with 70 v/v per cent ethanol), 5 times diluted with water. Curve II. Digitalis tincture, diluted with water and mixed with sodium hydroxide. Curve III. Aqueous digitalis macerate, after filtration, mixed with sodium hydroxide.

*Influence of Concentrations of 2:4-Dinitrodiphenylsulphone and Sodium Hydroxide on Maximum Extinction*

Solutions were made containing respectively 15, 30, 45, 60, 75 and 90 mg. of 2:4-dinitrodiphenylsulphone per 100 ml. The reaction was performed with 0·400 mg. of digitoxin (Uclaf). The concentration of the sodium hydroxide was 0·1 N (Figure 2) (curve 1), 0·25 N (curve 2) or 0·5 N (curve 3). Figure 2 shows that for each of the three concentrations of sodium hydroxide used the $E$ value increases with increase in concentration of 2:4-dinitrodiphenylsulphone up to a level of 75 mg./100 ml., but that only small increases in $E$ values are achieved with higher concentrations of the reagent. This is also confirmed by employing 9 ml. of this reagent instead of 4 ml. of ethanol and 5 ml. of reagent when the $E$ value is only slightly raised. At a concentration of 100 mg./100 ml. the solution is saturated. In the light of these findings a concentration of 75 mg./100 ml. was selected for this reagent.

It is also seen from Figure 2 that the concentration of sodium hydroxide influences colour density. It also influences the velocity of the reaction

and this was further investigated. An increase in normality above 0·15 does not increase the $E$ value but only increases the velocity of reaction as shown in Table I. Thus a concentration of the sodium hydroxide of 0·15 N is recommended.

*Absorption Curve*

The absorption curve was determined using 0·543 mg. of digitoxin (Uclaf), a concentration of 75 mg./100 ml. of 2:4-dinitrodiphenylsulphone

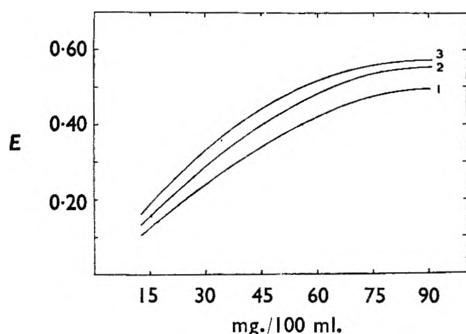


FIG. 2. The influence of the concentrations of 2:4-dinitrodiphenylsulphone and sodium hydroxide on maximum extinction. Curve 1. 0·10 N sodium hydroxide. Curve 2. 0·25 N sodium hydroxide. Curve 3. 0·50 N sodium hydroxide.

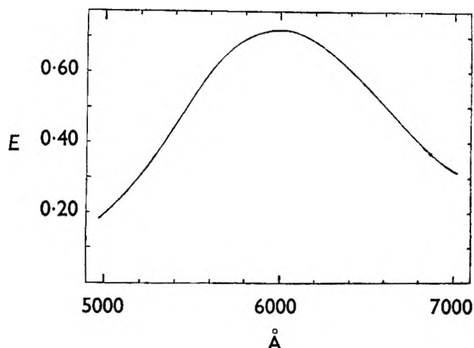


FIG. 3. The absorption spectrum of 0·543 mg. digitoxin. Concentration of 2:4-dinitrodiphenylsulphone 75 mg./100 ml.; 0·15 N sodium hydroxide.

and 0·15 N sodium hydroxide, extinctions were determined each 100 Å or, if necessary, each 50 Å. From Figure 3 it is seen that maximum colour density is obtained at a wavelength of 6,000 Å.

*Variations in the Blank*

The stability of the blank over a period of 2 hours was determined by measuring the colour density of a mixture of 4 ml. of ethanol, 5 ml. of reagent and 1 ml. of 0·15N sodium hydroxide against a mixture of 9 ml.

TABLE I

THE INFLUENCE OF THE NORMALITY OF SODIUM HYDROXIDE ON THE REACTION BETWEEN 2:4-DINITRODIPHENYLSULPHONE AND DIGITOXIN

| Normality of sodium hydroxide | Maximal extinction | Attained after 'minutes' | Remains constant 'minutes' |
|---|---|---|---|
| 0·05 | $0·40^5$ | 9 | 2 |
| 0·10 | $0·45^5$ | 6·5 | 1 |
| 0·15 | 0·48 | 5·5 | 1 |
| 0·20 | $0·48^5$ | 3·5 | 0·5 |
| 0·25 | 0·49 | 2·5 | – |

of ethanol and 1 ml. of 0·15N sodium hydroxide, at 6,000 Å in a 1·0 cm. cell. Table II shows that the colour of the blank does not change significantly and after 2 hours its value has little influence on results.

*Stability of Reagent*

From Table III it appears that the reagent can be stored in the dark for several days without deterioration.

*Influence of Water*

The same amount of digitoxin was dissolved in 17·5, 25·0, 50·0, 75·0 and 96·0 per cent ethanol respectively and the colour reaction was carried out as described. Table IV shows that there is only about a 5 per cent decrease in value of $E$ when the ethanol level of the glycoside solution is reduced from 96 to 17·5 per cent. At the lower level of alcohol the time required for development of maximum colour is increased by 1 minute.

TABLE II

THE INFLUENCE OF THE TIME ON THE COLOUR OF THE BLANK

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Extinctions ($\times 10^3$) | 0 | 0 | 2 | 4 | 5 | 6 | 7 | 9 | 9 | 10 | 10 | 10 |
| Measured after (minutes) | 1 | 5 | 10 | 15 | 20 | 25 | 30 | 40 | 60 | 80 | 100 | 120 |

*Influence of Temperature*

Solutions were maintained in a thermostat for 45 minutes at temperatures of 15°, 20°, 25° and 30°, the reaction mixture, when prepared, was allowed to stand for 1 minute at the same temperature and the extinction was determined immediately. Results are given in Table V. It will be seen that the maximum $E$ value decreases with increase of temperature, but that the time of attaining that maximum is decreased. It is recommended that the reaction be carried out at a temperature of 20°, and that higher temperatures be avoided.

TABLE III

THE INFLUENCE OF THE TIME ON THE REAGENT

| | | | | | | |
|---|---|---|---|---|---|---|
| Extinctions | 0·48 | $0{\cdot}47^8$ | $0{\cdot}47^8$ | 0·47 | 0·47 | 0·48 |
| After days | 0 | 1 | 3 | 4 | 6 | 11 |

TABLE IV

THE INFLUENCE OF WATER ON THE EXTINCTION

| Ethanol v/v per cent | Extinction | Maximum attained after (minutes) |
|---|---|---|
| 17·5 | $0{\cdot}45^8$ | 6·5 |
| 25·0 | 0·47 | 6·5 |
| 50·0 | $0{\cdot}47^8$ | 6 |
| 75·0 | $0{\cdot}47^8$ | 5·5 |
| 96·0 | 0·48 | 5·5 |

*Method of Estimation*

It was concluded from the foregoing that the estimation of digitalis glycosides should be carried out as follows. The glycoside or aglycone is dissolved in 4 ml. of ethanol, 5 ml. of a 0·075 per cent solution of dinitrodiphenylsulphone in ethanol is added followed by 1 ml. of 0·15N sodium hydroxide. The colour which develops is measured in a 0·5 cm.

cell at a wavelength of 6,000 Å against a blank of 4 ml. of ethanol with 5 ml. of reagent and 1 ml. of 0·15N sodium hydroxide. Spectral band width 45 Å; temperature of reaction must be maintained at 20°. The time required for the development of maximum colour intensity is not the same for some of the digitalis glycosides.

The reaction was applied to the estimation of digitoxin (Merck), digitoxigenin (Hoffmann–La Roche) and gitoxigenin (Sandoz). The same samples were also examined after boiling with acid in the following

TABLE V

THE INFLUENCE OF THE TEMPERATURE ON THE REACTION

| Temperature °C. | Extinction | |
|---|---|---|
| | Time of development (minutes) | Maximum |
| 15·0 | 7 | 0·51$^{0}$ |
| 20·0 | 5·5 | 0·49$^{8}$ |
| 25·0 | 4·5 | 0·47$^{6}$ |
| 30·0 | 3·5 | 0·45$^{2}$ |

manner; the glycoside or aglycone is dissolved in 5 ml. of ethanol, 2 ml. of 4N HCl and 13 ml. of water added (final acid concentration 0·4N) and the mixture is boiled under reflux for 30 minutes. The contents of the flask are then transferred to a separating funnel, the reflux condenser and flask washed twice with 5 ml. of 25 per cent ethanol which are also transferred to the separating funnel. The acid product and washings are then shaken out with three successive quantities each of 22·5 ml. of chloroform, each shaking being for 1 minute. The combined chloroform extracts are dried with exsiccated sodium sulphate, after which they are filtered, the filter and flask washed with 5 ml. of dry chloroform and the solvent is distilled off.

It was found that the extinction coefficients of the two aglycones were increased by this process of boiling with acid. Smithuis[10] has shown that this increase is connected with the formation of anhydro compounds; gitoxigenin is converted into dianhydrogitoxigenin and digitoxigenin is converted into a monoanhydro compound.

When graphs were plotted of colour extinctions against glycoside or aglycone concentrations of solutions used, they were found to be straight lines for digitoxin and for digitoxigenin whether pre-treated with acid or not. Gitoxigenin, if pre-treated with acid also gave a straight line; but if not so pre-treated the graph was a curve with molar extinctions diminishing from 12,500 to 10,700.

Maximum colour intensity is developed after the addition of the sodium hydroxide in 5·5 minutes for digitoxin, in 4·5 minutes for digitoxigenin and in 3 minutes for these two substances or for gitoxigenin after acid treatment. The maximal colour value is constant for 1 or 1½ minutes.

Table VI records the extinction coefficients and molar extinctions of some digitalis glycosides with this new reagent. These values are also compared with those obtained when using other quantitative reagents, viz. picric acid (Baljet reagent), 3:5-dinitrobenzoic acid (Kedde reagent) and *m*-dinitrobenzene (Raymond reagent). Standard deviations for the new

reagent are also recorded. The Tattje modification of the 3:5-dinitrobenzoic acid process[11] yields a higher value for digitoxin and this is also indicated.

TABLE VI

EXTINCTION COEFFICIENTS AND MOLAR EXTINCTIONS OF SOME DIGITALIS GLYCOSIDES OBTAINED WITH 2:4-DINITRODIPHENYLSULPHONE, COMPARED WITH THOSE OBTAINED WITH PICRIC ACID, 3:5-DINITROBENZOIC ACID AND *m*-DINITROBENZENE

| Glycoside of aglycone | Treatment | 2:4-Dinitrodiphenylsulphone | | Picric acid | | 3:5-Dinitrobenzoic acid | | *m*-Dinitrobenzene |
|---|---|---|---|---|---|---|---|---|
| | | *E* (1 per cent, 1 cm.) | Mol. ext. | *E* (1 per cent, 1 cm.) | Mol. ext. | *E* (1 per cent, 1 cm.) | Mol. ext. | Mol. ext. |
| Digitoxin . . . . | none | 303<br>s. d.= 3·22 | 23,200<br>s. d. =246 | 222·5<br>190[13] | 17,000<br>14,500[13] | 111[11]<br>77.8[12]<br>77·5[14] | 8500[11]<br>5940[12] | 11,110[9]<br>14,700[13] |
| Digitoxin . . . . | boiled with acid | 370<br>s. d.= 2·73 | 28,300<br>s. d.= 209 | 230 | 17,600 | 106·6[12] | 8140[12] | |
| Digitoxigenin . . | none | 653<br>s. d.= 6·33 | 24,400<br>s. d.= 237 | 460 | 17,200 | 169·8[12]<br>255 | 6360[12]<br>9540 | 10,720[14] |
| Digitoxigenin . . | boiled with acid | 778<br>s. d.= 4·55 | 29,100<br>s. d.= 170 | 486 | 18,200 | 230·9[12]<br>329 | 8640[12]<br>12,300 | |
| Gitoxin . . . . | boiled with acid | 232 | 18,100 | 200 | 15,600 | 100·1[12] | 7810[12] | |
| Gitoxigenin . . | none | ca. 314 | | 346 | 13,500 | 187 | 7290 | 3920[9] |
| Gitoxigenin . . | boiled with acid | 464<br>s. d.= 3·29 | 18,100<br>s. d.= 128 | 379 | 14,800 | 286 | 11,100 | |

It is seen from Table VI that the 2:4-dinitrodiphenylsulphone reagent is much more sensitive than are the other reagents, especially in the case of digitoxin and digitoxigenin, but also to a lesser extent for gitoxigenin.

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# THE EFFECT OF A STANDARDISED SENNA PREPARATION ON THE HUMAN BOWEL

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A standardised senna preparation, was administered to 52 ward patients, in doses to each person of 1, 2, 3 and 4 tablets, and to 126 medical students in a dose of 3 tablets. Three active tablets and 3 inert tablets were also administered to each of 99 students, and one active tablet and one inert tablet to each of 44 students. With 3 tablets of senna preparation the "speed of action" with students (mean 12·15 hours) was significantly slower than with ward patients (mean 9·7 hours). In the ward patients the frequency of griping, of looseness of stool and of multiple bowel movements increased with rising dosage. In the students' trial with one tablet there was no significant difference between the results with the active and with inert tablets; one senna tablet would appear to have a negligible pharmacological effect. Thirty-nine students and 5 ward patients experienced griping in the absence of other arbitrarily defined evidence of overdosage. The incidence of anorexia and nausea is as high with inert as with active tablets. Analysis of the results showed no significant difference in response between male and female students.

Senna belongs to the anthraquinone group of purgatives and owes the greater part of its activity to two anthranol-containing glycosides which have been named sennosides A and B. In addition, other principles including a third glycoside are present and reinforce the action of the sennosides[1]. The pharmacology of senna is well reviewed by Abrahams[2]. After ingestion the sennosides are absorbed and pass into the blood stream. After chemical changes have occurred, the active principles, the emodins, reach the large bowel. Here, the propulsive movements are greatly stimulated, thus causing a rapid passage of faecal contents.

The standardised senna preparation, prepared from Alexandrian senna pods, is presented as tablets, and is also available incorporated with cocoa, malt and sugar as "granules". Two batches of tablets were used in the work described. Each tablet in the first batch contained the "total active constituents" of 250 mg. Alexandrian senna pod, B.P.: the sennoside A + B content of each tablet by chemical assay was 7·50 mg. Each tablet in the second batch contained the "total active constituents" of 200 mg. Alexandrian senna pod, B.P.: the sennoside A + B content of each of these tablets was 7·25 mg. Tablets of both batches were regarded as having the same potency for clinical purposes.

The object of the present trial was to assess the effect on bowel function, to observe the incidence and type of side-effects, and to make similar observations after giving inert tablets. The trial was conducted in two parts: in the first the drug was administered to ward patients, in the second to volunteer medical students.

The ward series consisted of 52 male patients, selected at random, but excluding the seriously or acutely ill, those with severe chronic constipation

requiring enemata, and those over 80 years. Their normal frequency of bowel movement is shown in Table I, and their normal stool consistence in Table II. At 8 a.m., immediately after breakfast, each patient received one tablet of 7·50 mg., and the following observations were made. (*a*) Time

TABLE I

NORMAL FREQUENCY OF BOWEL MOVEMENT—WARD PATIENTS AND STUDENTS

| Frequency of movement | Number of patients | Number of students |
|---|---|---|
| Twice daily.. .. .. .. | 4 | 32 |
| Once daily .. .. .. .. | 8 | 188 |
| Twice in three days .. .. | 10 | 26 |
| Every second day .. .. .. | 16 | 23 |
| Less often than every second day.. | 14 | — |

required for the bowels to move; (*b*) the number of bowel movements in the 23 hours after administration of the drug; (*c*) bulk, consistence and colour of the stools, and (*d*) side-effects.

This regimen was later repeated at intervals of at least 3 days using 2, 3 and 4 tablets.

## RESULTS

*Time of action.* The time taken to act is shown in Table III(*a*).

*Consistence of stool.* None of the patients in the trial gave a history of recent loose bowel movements. Stool consistence in relation to dose is shown in Table IV(*a*).

TABLE II

NORMAL STOOL CONSISTENCE—WARD PATIENTS AND STUDENTS

| | Firm | Soft | Loose |
|---|---|---|---|
| Number of patients .. .. | 42 | 10 | — |
| Number of students .. .. | 211 | 58 | — |

*Number of bowel movements.* No patient had more than four bowel movements in the 23 hours after the administration of the tablets in any dosage. Details are set out in Table V(*a*).

*Bulk and colour of the stools.* No changes in bulk or colour of the stools were noted.

*Side-effects.* Two patients had anorexia after 4 tablets, one patient after 3 tablets. None experienced nausea or vomiting.

*Griping.* Results are set out in Table VI(*a*). Of those who had griping with the larger doses, 4 and 3 tablets, 5 in each case had not more than two bowel movements, neither of which was loose. No other side-effects were noted.

### *Trial with Student Volunteers*

This was in three stages, with a total of 208 male and 61 female volunteers, with ages from 19 to 35 years: all but 68 were under 23 years. The tablets were swallowed immediately before retiring to bed and

observations were recorded on a form. The normal frequency of bowel movement is shown in Table I, and the normal stool consistence in Table II. None of the students had used a laxative regularly, and only 9 of the 208 men and 4 of the 61 women had used a laxative intermittently. In the first stage, 3 tablets (7·50 mg.) were administered to 96 male and 30 female students.

## RESULTS

*Time of action.* This is shown in Table III(*b*). One woman and 5 men were awakened from sleep by the call to stool. Six women and 29 men had a first bowel movement before breakfast.

TABLE III

MEAN INTERVALS BETWEEN MEDICATION AND FIRST BOWEL MOVEMENT

| | | Mean interval hours | S.D. |
|---|---|---|---|
| | *Ward trial:* | | |
| (*a*) | Senna preparation tablets (7·50 mg.) | | |
| | 1 tablet | 12·8 | 9·43 |
| | 2 tablets | 12·2 | 8·01 |
| | 3 " | 9·7 | 6·43 |
| | 4 " | 8·6 | 5·53 |
| | *Student trials:* | | |
| (*b*) | 3 senna preparation tablets (7·50 mg.) | | |
| | 96 men | 11·79 | 2·74 |
| | 30 women | 11·63 | 6·41 |
| | Total 126 students | 11·75 | 4·02 |
| | 3 senna preparation tablets (7·25 mg.) | | |
| (*c*) | 75 men | 12·49 | 5·2 |
| | 24 women | 13·12 | 10·6 |
| | Total 99 students | 12·65 | 6·8 |
| (*d*) | 3 inert tablets 99 students | 17·18 | 11·96 |
| (*e*) | 1 senna preparation tablet (7·25 mg.) | | |
| | 44 students | 16·05 | 8·51 |
| (*f*) | 1 inert tablet 44 students | 15·75 | 10·04 |

*Consistence of stool and number of bowel movements.* The consistence of the first stool is shown in Table IV(*b*): in none was the stool firmer than normal. In addition to those whose first stool was loose, 30 men and 14 women whose first stool was firm or soft had a second or subsequent loose stool: thus 67 men, 70 per cent, and 19 women, 63 per cent, had at least one loose stool after the tablets. The number of bowel movements experienced in the 23 hours after the senna preparation is shown in Table V(*b*).

*Bulk and colour of the stools.* No change in stool colour was noticed. Of the women, 22 noticed no change in bulk of the first stool, 6 thought it was increased, and 2 reported a decrease. Fifty-seven men noticed no change in bulk of the first stool, 29 thought the bulk was increased and 10 thought it decreased.

*Side-effects.* Three female and 7 male students experienced anorexia; 4 female and 8 male felt nauseated; and one male student vomited during the day after taking the drug although he attributed the vomiting to dietary indiscretion.

*Griping.* Results are set out in Table VI(*b*). There was no correlation of griping and increased bowel evacuations nor of griping and the occurrence of loose motions. But, of the 51 men and 20 women who experienced mild griping, 5 men and 2 women had one or two bowel movements only, neither of which was loose; and of the 15 men and 3 women who experienced moderate or severe griping, 7 men and 3 women similarly had one or two bowel movements only, neither of which was loose.

In the second and third stages of the trial with students, inert control tablets, identical in apperarance, but composed of powdered dried grass, sugar, starch and cocoa, were used. The students were told that all the tablets used were active, but of different batches, a deliberate departure from fact which was felt to be in the interests of scientific control.

TABLE IV

STOOL CONSISTENCE WITH VARYING MEDICATION

| | | Number of subjects whose first movement after medication was | | | Total No. who had a loose bowel movement at any time in the following 24 hours |
|---|---|---|---|---|---|
| | | Firm | Soft | Loose | |
| | *Ward trial:* | | | | |
| (*a*) | Senna preparation tablets (7·50 mg.) | | | | |
| | 1 tablet | 35 | 13 | 4 | 6 |
| | 2 tablets | 8 | 26 | 18 | 20 |
| | 3 " | 8 | 18 | 26 | 29 |
| | 4 " | 0 | 22 | 30 | 34 |
| | *Student trials:* | | | | |
| (*b*) | 3 senna preparation tablets (7·50 mg.) | | | | |
| | 96 men | 20 | 39 | 37 | 67 |
| | 30 women | 11 | 14 | 5 | 19 |
| | Total 126 students | 31 | 53 | 42 | 86 |
| (*c*) | 3 senna preparation tablets (7·25 mg.) | | | | |
| | 75 men | 17 | 38 | 30 | 45 |
| | 24 women | 3 | 13 | 8 | 17 |
| | Total 99 students | 20 | 51 | 38 | 62 |
| (*d*) | 3 inert tablets 99 students | 61 | 33 | 5 | 9 |
| (*e*) | 1 senna preparation tablet (7·25 mg.) 44 students | 24 | 17 | 3 | 7 |
| (*f*) | 1 inert tablet 44 students | 32 | 10 | 2 | 5 |

In the second stage each of 99 students (75 men and 24 women) received, in a random order, 3 senna preparation tablets (7·25 mg.) and 3 inert tablets at an interval of a week.

## RESULTS

*Time of action.* This is shown in Table III(*c*) and (*d*). With the senna preparation no student was awakened from sleep by the call to stool: with the inert tablets, 2 men were awakened from sleep.

*Consistence of stool.* The consistence of the first stool after the senna preparation and inert tablets is shown in Table IV(*c*) and (*d*). With the

senna preparation, in addition to those whose first stool was loose, 13 men and 9 women whose first stool was firm or soft had a second or subsequent loose stool: and with the inert tablets, 4 men whose first stool was firm or soft had a second or subsequent loose stool: thus 50 students had at least one loose stool after the active preparation, and 9 students recorded a similar effect after the inert tablets.

*Number of bowel movements.* The number of bowel movements experienced in the 23 hours after the senna preparation and inert tablets is shown in Table V(*c*) and (*d*).

TABLE V

NUMBER OF BOWEL MOVEMENTS IN THE 23 HOURS AFTER MEDICATION

| | After | Number of subjects with varying number of bowel movements | | | | |
|---|---|---|---|---|---|---|
| | | 1 movement | 2 movements | 3 movements | 4 movements | More than 4 movements |
| | *Ward trial:* | | | | | |
| (*a*) | Senna preparation tablets (7·50 mg.) | | | | | |
| | 1 tablet | 43 | 6 | 3 | — | — |
| | 2 tablets | 31 | 16 | 3 | 2 | — |
| | 3 " | 17 | 23 | 7 | 5 | — |
| | 4 " | 12 | 20 | 13 | 6 | 1 |
| | *Student trials:* | | | | | |
| (*b*) | 3 senna preparation tablets (7·50 mg.) | | | | | |
| | 96 men | 25 | 44 | 18 | 7 | 2 |
| | 30 women | 6 | 6 | 10 | 4 | 4 |
| | Total 126 students | 31 | 50 | 28 | 11 | 6 |
| (*c*) | 3 senna preparation tablets (7·25 mg.) | | | | | |
| | 75 men | 27 | 29 | 12 | 4 | 3 |
| | 24 women | 3 | 8 | 7 | 4 | 2 |
| | Total 99 students | 30 | 37 | 19 | 8 | 5 |
| (*d*) | 3 inert tablets | 62 | 28 | 5 | 3 | 1 |
| (*e*) | 1 senna preparation tablet (7·25 mg.) | 26 | 12 | 4 | 2 | — |
| (*f*) | 1 inert tablet | 32 | 12 | — | — | — |

*Bulk and colour of the stools.* No change in stool colour was noticed. With the senna tablets, 37 men and 14 women noticed no change in the bulk of the stools: 28 men and 3 women thought it increased, and 10 men and 7 women thought it decreased. With inert tablets, 51 men and 17 women noted no change in the bulk of the stools, 9 men and 2 women thought it was increased, and 15 men and 5 women thought it had decreased.

*Side-effects.* With the senna preparation, 7 men and 2 women experienced anorexia: 3 men and one woman felt nauseated. With the inert tablets, 8 men experienced anorexia, and 2 men felt nauseated: no woman had anorexia or nausea.

*Griping.* The results, with the senna preparation and with inert tablets, are set out in Table VI(*c*) and (*d*). Of the 40 men and 17 women who experienced mild or moderate griping after the senna preparation, 21 men and one woman had one or two bowel movements only, neither of which was loose.

In the third stage each of 44 students, 37 men and 7 women, received, in a random order, one senna preparation tablet (7·25 mg.) and one inert tablet at an interval of a week.

## Results

*Time of action.* This is shown in Table III (*e*) and (*f*).

*Consistence of stool.* This is shown in Table IV(*e*) and (*f*).

*Number of bowel movements.* This is shown in Table V(*e*) and (*f*).

*Side-effects.* With the senna preparation, one student felt nauseated and vomited.

*Griping.* With the senna preparation 7 students had mild griping and one had moderate griping. There was no griping after the inert tablets.

TABLE VI

Incidence of griping

| | After | Griping absent | Mild griping | Moderate griping | Severe griping |
|---|---|---|---|---|---|
| | *Ward trial:* | | | | |
| (*a*) | Senna preparation tablets (7·50 mg.) | | | | |
| | 1 tablet .. .. | 49 | 3 | — | — |
| | 2 tablets .. .. | 40 | 10 | 2 | — |
| | 3 " .. .. | 38 | 9 | 4 | 1 |
| | 4 " .. .. | 31 | 15 | 4 | 2 |
| | *Student trials:* | | | | |
| (*b*) | 3 senna preparation tablets (7·50 mg.) | | | | |
| | 96 men .. .. | 30 | 51 | 10 | 5 |
| | 30 women .. .. | 7 | 20 | 3 | — |
| | Total 126 students.. .. | 37 | 71 | 13 | 5 |
| (*c*) | 3 senna preparation tablets (7·25 mg.) | | | | |
| | 75 men .. .. | 30 | 28 | 12 | 5 |
| | 24 women .. .. | 6 | 7 | 10 | 1 |
| | Total 99 students .. | 36 | 35 | 22 | 6 |
| (*d*) | 3 inert tablets 99 students .. | 85 | 14 | — | — |
| (*e*) | 1 senna preparation tablet (7·25 mg.) 44 students .. | 37 | 7 | — | — |
| (*f*) | 1 inert tablet 44 students .. | 44 | — | — | — |

## Discussion

To ensure accuracy of dosage in this trial, tablets were used rather than granules. The dosage of 3 tablets used in the majority of the trials with students was chosen to represent the middle of the dose range (2–4 tablets) suggested by the manufacturers. It was thought that a more accurate record of the side-effects would be obtained if the drug was administered to ward patients *in the morning.*

Most subjects (patients and students) did not suffer from "constipation". However, habitual constipation is not in fact a "disease" as the term is ordinarily understood, and there is no evidence that colonic motility is abnormal in persons suffering from constipation, which results from chronic failure to move the bowels when the rectum is loaded.

Comparison of the results obtained with 7·50 mg. and 7·25 mg. tablets with different groups of students shows no significant differences. None of the 269 volunteers uses a laxative regularly, and only 13 (9 men and

4 women) use a laxative occasionally. This presents an interesting contrast to the conclusion published by Reid[3] that 73 of 565 (12·9 per cent) boys and girls of age 15 years took laxatives at least once weekly, figures which may reveal the influence of the parents.

*Psychological Factors*

Comparison of the results obtained in the controlled trial with students with one standardised senna preparation and one inert tablet shows no significant difference. It appears that one tablet of the senna preparation does not exercise a significant pharmacological effect. Three inert tablets produced a slightly greater incidence of multiple bowel movements and of griping than one inert tablet, but the differences are not significant.

Comparison of 3 senna preparation tablets and 3 inert tablets for "speed of action", multiplicity and looseness of stool, and griping, gives differences which are highly significant.

*"Speed of Action" of the Senna Preparation*

The mean "speed of action" of 3 tablets of the senna preparation with ward patients was 9·7 hours, but the mean value among 225 students was 12·15 hours (11·75 hours and 12·65 hours in the two groups of students). This difference (2·45 hours) is significant, and can be attributed to the differing times of administration, and to the age-scatter, activity and diets of the two groups of subjects. These and other factors (anxieties, trivial gastrointestinal infections) were no doubt at work to modify the speed of action, but similar factors would obtain in the clinical use of the drug.

Within each group of subjects there is a wide variation in the "speed of action". The results shown in Table III support the conclusion reached in a previous communication[4] that the speed of action of senna quoted by Clark[5] and by Goodman and Gilman[6] as under 6 hours, and after 8–10 hours respectively, is too short, particularly if applied to healthy people.

*Stool Consistence*

A laxative for routine use should produce a soft rather than a loose stool. In 148 of 225 students who received 3 senna preparation tablets one or more stools were loose. Among 52 ward patients who, on different occasions received doses of 1, 2, 3 and 4 tablets, there was a progressively higher frequency of occurrence of loose stools: the percentage of the group of 52 patients who passed one or more loose stools was respectively 12, 40, 60 and 66 per cent. This suggests that if looseness of bowel is to be avoided with this preparation the initial dose should be one tablet, the number being increased if necessary.

*Anorexia and Nausea*

The incidence of anorexia and nausea with 3 tablets is about 10 per cent: however, as the incidence after 3 inert tablets is very similar, these symptoms would appear to be psychological in origin.

*Griping*

The incidence of griping in the ward trial varied from 21 instances after 4 tablets, and 14 cases after 3 tablets (27 per cent), to 3 instances after one tablet.

In the students' trials with 3 tablets 152 students (67·5 per cent) experienced griping, which in 46 cases was moderate or severe.

This difference in the incidence of griping after 3 tablets in ward patients and students is a striking one, and can be attributed in part to the fact that ward patients can move their bowels whenever the call to stool arises, but students going about their normal daily routine must naturally postpone bowel evacuation until a suitable time. It is equally true that in the clinical use of the preparation such hazards of time and place have also to be faced. Griping may of course occur more frequently in students with normal bowel habit than in costive patients who have a higher threshold of bowel discomfort.

Thirty-nine students experienced griping in the absence of other manifestations of overdosage, which are arbitrarily defined as more than 2 bowel movements, or loose bowel movements. Five ward patients experienced griping in the absence of other evidence of overdosage after they had taken doses of 4 tablets and of 3 tablets.

With students and patients who receive one tablet, and with patients who receive 2 tablets, griping is unusual. However, some patients may require 3 tablets as a single dose to produce a laxative effect, and with 3 tablets some subjects experience griping without other evidence of overdosage. Unless griping is invariably the first symptom of overdosage to appear as dosage is progressively increased, the occurrence of griping without other evidence of overdosage conflicts with the view[7] that "the great advantage of prescribing a standardised preparation of constant laxative action is that, once the dose for each patient is decided, there should be no griping since this seems to be a symptom of overdosage".

*Sex Differences*

There are no significant differences between the results obtained with men and women students.

*Acknowledgements.* I am indebted to the patients and students who submitted to the tests. Thanks are due to Dr. A. W. Lees for permission to include in this series patients under his care: and to Professor S. Alstead and Dr. T. J. Thomson for their advice and encouragement. Messrs. Westminster Laboratories kindly supplied the Senokot and inert tablets.

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# THE MODE OF ACTION OF CHLORINATED BISPHENOL ANTIBACTERIALS

## PART I. METAL CHELATES OF HEXACHLOROPHENE AND THIOBISDICHLORPHENOL


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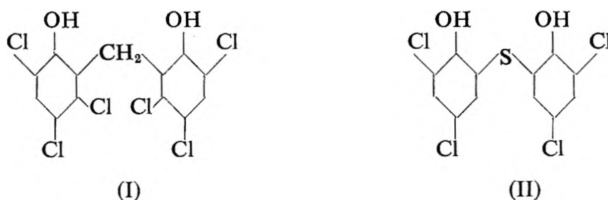
2:2′-Thiobis(4:6-dichlorphenol) has been found to chelate with $Fe^{++}$, $Fe^{+++}$, $Cu^{++}$, $Mn^{++}$ and $Co^{++}$ as shown by colorimetric, potentiometric and analytical data. Crystalline copper, iron and cobalt chelates of thiobisdichlorphenol have been isolated and their structures determined. Hexachlorophene chelates with $Fe^{++}$, $Fe^{+++}$ and $Cu^{++}$ as demonstrated by potentiometric titrations in the presence and absence of metals. But stable crystalline chelates could not be isolated. The suggestion is made that the structure activity relationship in the bisphenol group of antibacterials is connected with the ability of the molecule to chelate with certain metals.


OF the bis-phenol group of antibacterials, hexachlorophene (2:2′-methylenebis (3:4:6-trichlorphenol), I) and 2:2-′thiobis (4:6-dichlorphenol) (II), have been found to possess high antibacterial activity[1-3].




Structural requirements for maximum activity in this group are (i) the linkage of the two rings must be in the 2:2′-position to the hydroxyl groups, (ii) this linkage should be direct or through a methylene, O or S group; linkages such as ethylene or higher alkyl chains or bulkier groups such as –CO– or $-SO_2-$ lead to marked loss in activity, (iii) both rings must contain halogen; the greater the substitution the higher the activity, (iv) substitution of the hydroxyl groups by formation of ethers, etc., leads to loss of activity[4].

Consideration of these requirements coupled with the fact that the above two compounds are bacteriostatic against certain Gram-positive bacteria at concentrations of about 1 in 2 million, suggested that a highly specific action was involved. Molecular models (Courtaulds) of the two compounds were constructed. Rotation of the two rings around the sulphur atom of thiobisdichlorphenol was possible, but a particular fixed structure could be favoured due to hydrogen bonding between the hydroxyl groups. Models of hexachlorophene indicated that only two structures (conformations) were possible due to rigidity conferred on

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the molecule by the chlorine atoms. In one of these structures (Fig. 1A) the two hydroxyls were in close proximity and in the other they were some distance apart. The proximity of the two hydroxyl groups was confirmed in both compounds by formation of methylene ethers by reacting the compounds with methylene bromide in boiling butanol containing two equivalents of sodium butoxide.

The $pK_a$ values of the two hydroxyl groups of hexachlorophene and thiobisdichlorphenol are $pK_1 5{\cdot}4$, $pK_2 10{\cdot}85$[5] and $pK_1 4{\cdot}82$, $pK_2 10{\cdot}50$[3] respectively. This again indicates close proximity of the hydroxyls and

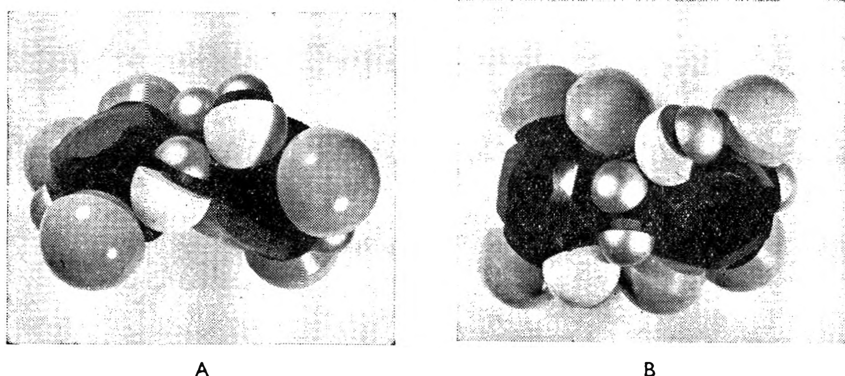



FIG. 1. Molecular models of hexachlorophene.

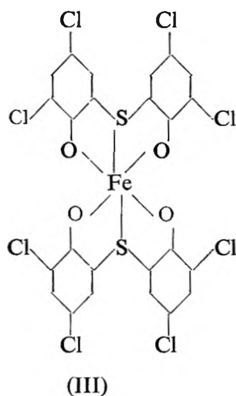
in addition at the physiological pH of 7·3 one hydroxyl group is completely ionised and the other almost completely unionised in both compounds. These facts suggested that metal chelation, involving the two hydroxyl groups, may be involved in the biological activity of these compounds. Potentiometric titration showed that both compounds chelated with certain metals. Thiobisdichlorphenol forms more stable chelates and some have been isolated in crystalline form.

## RESULTS

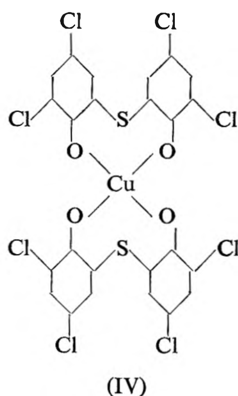
### *Structures of Thiobisdichlorphenol Chelates*

By the method of continuous variation introduced by Job,[6] it was possible to elucidate the combining ratios of thiobisdichlorphenol with metal ions. This method involves measurement of the colour produced by chelates when the ratio of metal to chelating agent is varied while at the same time keeping the total concentration the same. The point at which maximum colour is developed then represents the ratio at which the metal and chelating agent are combined in the chelate. Figure 2 indicates the combining ratios of thiobisdichlorphenol with $Fe^{++}$, $Fe^{+++}$ and $Cu^{++}$ respectively, in 70 per cent aqueous ethanol. Measurement was on a Hilger photometer with a 520 $m\mu$ filter.

$Fe^{++}$ and $Fe^{+++}$ with co-ordination numbers of 6 are seen to form chelates with a 1 to 2 ratio of metal to chelating agent. Their structures would be represented by III.



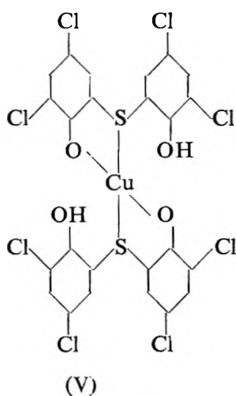

(III)



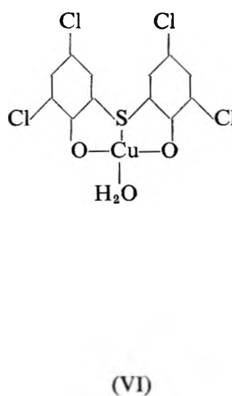

(IV)

The ferrous chelate would carry a double negative charge and the ferric chelate a single negative charge.

For copper (co-ordination number 4) one maximum and a point of inflection are obtained, Figure 2. The maximum corresponds to a ratio of Cu to chelating agent of 1 to 2 and the point of inflection to a 1 to 1 combination. Evidently two chelates are formed, the possible structures being either IV or V for the 1 : 2 chelate and VI for the 1 : 1 chelate.




(V)




(VI)

Although Sidgwick[7] has pointed out that oxygen has a greater affinity for copper than sulphur in chelates, the fact that a five-membered ring involving S and O is formed in V and not in IV, favours V as the most likely structure.

The influence of pH on the stability of these chelates, measured by optical density at 520 mμ in 70 per cent ethanol is shown in Figure 3. The top curve was obtained with a total volume of 4 ml. of 0·039M solutions of ferrous sulphate and thiobisdichlorphenol diluted to 100 ml. For copper, total volumes of 8 ml. of 0·039M solutions of copper sulphate and thiobisdichlorphenol were diluted to 100 ml.

Maximum stability is seen to occur in or about the physiological pH range.

*Demonstration of Chelation by Potentiometric Titration*

Hexachlorophene was found to chelate with $Fe^{++}$, $Fe^{+++}$ and $Cu^{++}$ but not with $Co^{++}$, $Mn^{++}$ or $Mg^{++}$. Thiobisdichlorphenol formed chelates with all these metals with the exception of magnesium. Potentiometric titration both in the presence and absence of metals was used to demonstrate chelation. Figure 4 shows the titration of thiobisdichlorphenol M/200 against 0·1N NaOH in the presence and absence of ferrous

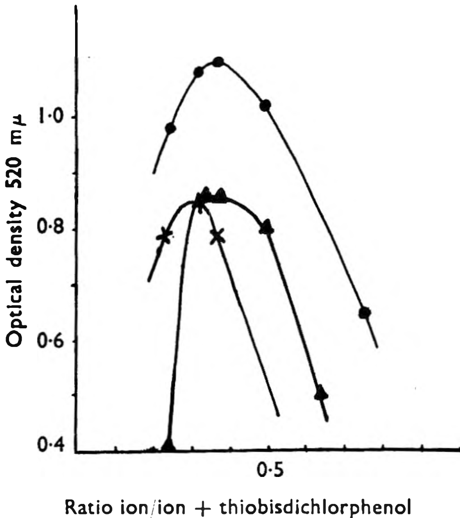



FIG. 2. Combining ratios of $Fe^{++}$, $Fe^{+++}$ and $Cu^{++}$ with thiobisdichlorphenol carried out in 70 per cent aqueous ethanol. 4 ml. total volumes of 0·039M solutions of thiobisdichlorphenol and iron salts made up to 100 ml. A total volume of 8 ml. of 0·039M solutions were used in the case of copper and made up to 100 ml. Adjustment of pH made with 5 per cent NaOH.

●—● $Fe^{++}/Fe^{++}$ + thiobisdichlorphenol pH 6·5.
×—× $Fe^{+++}/Fe^{+++}$ + thiobisdichlorphenol pH 6·0.
▲—▲ $Cu^{++}/Cu^{++}$ + thiobisdichlorphenol pH 7·5.

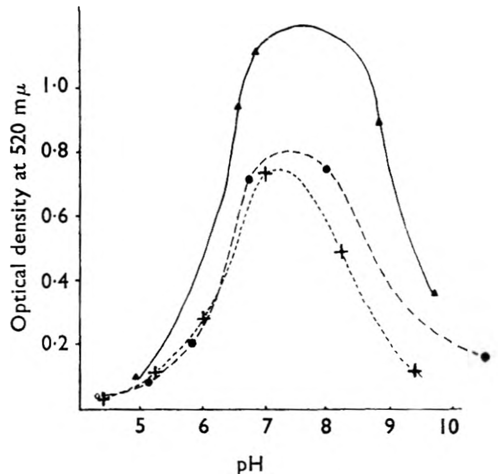



FIG. 3. Influence of pH on the stability of metal chelates of thiobisdichlorphenol as measured by the optical density at 520 mμ in 70 per cent ethanol solution. The top curve was obtained using a total volume of 4 ml. of 0·039M solutions of ferrous sulphate and chelating agent then diluting to 100 ml. In the case of copper, total volumes of 8 ml. of 0·039M solutions of copper sulphate and chelating agent were diluted to 100 ml.

▲——▲ $FeSO_4$ + thiobisdichlorphenol mixture 1:1.
●- - -● $CuSO_4$ + thiobisdichlorphenol mixture 1:2.
× - - - × $CuSO_4$ + thiobisdichlorphenol mixture 1:1.

sulphate M/400 and copper sulphate in 70 per cent ethanol. Hexachlorophene gives similar curves.

Chelation takes place in each instance, but is more marked with thiobisdichlorphenol. Deep red brown colours were formed in both titrations of hexachlorophene in the presence of iron or copper. With copper a maximum colour was produced at about pH 6·5, but at pH 7·5 the colour was discharged and at increasing pH a pale green solution was formed. The deep red brown colour formed with hexachlorophene and ferrous sulphate at maximum concentration at pH 6, was followed

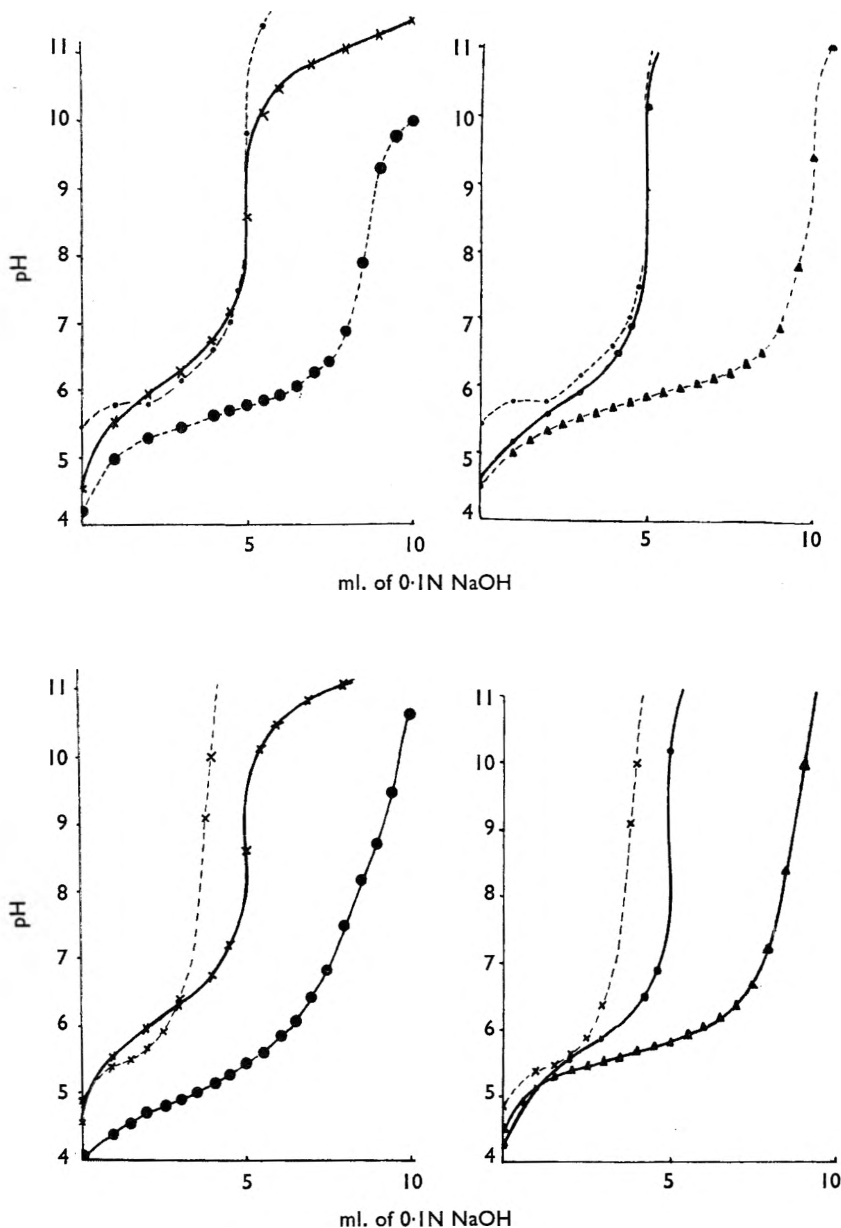
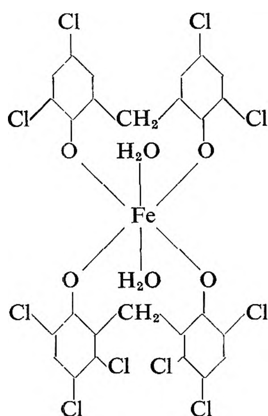



FIG. 4. Illustration of chelation of thiobisdichlorphenol and hexachlorophene with ferrous sulphate and copper sulphate by potentiometric titration with 0·1N NaOH in 70 per cent ethanol.
•---• M/400 $FeSO_4$; ×——× M/200 of thiobisdichlorphenol; ●---● M/200 thiobisdichlorphenol + M/400 $FeSO_4$; •——• M/200 hexachlorophene; ▲---▲ M/200 hexachlorophene + M/400 $FeSO_4$; ×---× M/400 $CuSO_4$; ●——● M/200 thiobisdichlorphenol + M/400 $CuSO_4$; ▲——▲ M/200 hexachlorophene + M/400 $CuSO_4$.

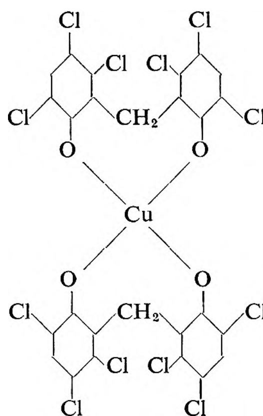
by a loss in colour intensity at pH 7·8. At higher pH an orange brown colour was formed. Chelation was also demonstrated on titration of hexachlorophene in the presence of ferric alum or ferric chloride.

Thiobisdichlorphenol formed claret coloured solutions with ferrous sulphate and copper sulphate. With cobalt chloride a pink solution was formed initially followed by a white precipitate of the cobalt chelate. Figure 5 shows the titration of hexachlorophene and cobalt chloride. No chelation has taken place, the curve for hexachlorophene in the presence of cobalt chloride being compounded of the two original curves.

The possible structures of the hexachlorophene chelates with iron and copper are VII and VIII.




(VII)




(VIII)

Stability of constants of these chelates have not been determined.

*Isolation of Crystalline Chelates*

Highly crystalline chelates of thiobisdichlorphenol and copper and thiobisdichlorphenol and iron ($Fe^{++}$) were isolated by bringing the metal and chelating agent together in 70 per cent ethanol solution, adjusting to the pH for maximum stability with NaOH and diluting with an equal volume of water. The $Fe^{++}$ chelate formed a thick gel on cooling and on standing violet-black micro needles were deposited. The copper chelate formed light brown needles. $Fe^{+++}$ formed black needles which were contaminated with crystals of the chelating substance. Analysis of the $Fe^{-+}$ chelate gave results corresponding to a 1 : 2 chelate which confirms the structure indicated previously. The copper chelate analysed for a 1 : 1 chelate which is represented by structure VI. Evidently this structure is more insoluble in more aqueous media. By reacting a solution of the chelating agent in 0·1N NaOH with 1 mole equivalent of a solution of copper sulphate, a brown precipitate was formed which analysed more closely to VI than to V, although the compound was impure probably due to the presence of copper oxide. The $Fe^{++}$ chelate was also formed by reacting a solution of thiobisdichlorphenol in 0·1N

NaOH with 0·5 mole equivalent of ferrous sulphate solution. A deep claret coloured solution was formed from which the chelate was isolated by extraction with chloroform. The cobalt chelate was isolated as a semi-crystalline precipitate during the potentiometric titration of M/200 thiobisdichlorphenol in the presence of M/400 $CoCl_2$. It could be purified by recrystallisation from ethanol. Analysis showed it to

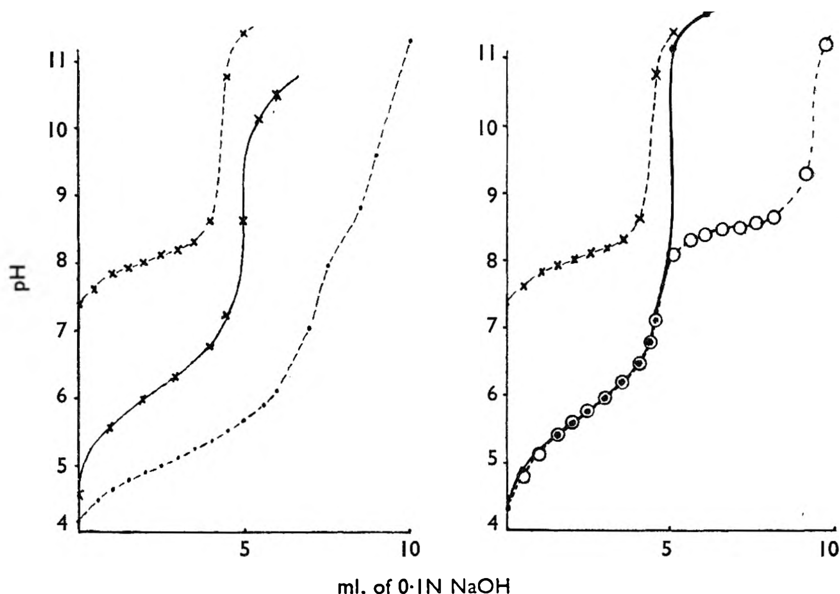



FIG. 5. Potentiometric titration of thiobisdichlorphenol and hexachlorophene with 0·1N alkali in the presence and absence of cobalt chloride.
×–×–× M/400 $CoCl_2$; ×——× M/400 thiobisdichlorphenol; •- - -• M/200 thiobisdichlorphenol + M/400 $CoCl_2$; •——• M/200 hexachlorophene; O - - O M/200 hexachlorophene + M/400 $CoCl_2$.

be a 1:2 chelate containing 7 moles of water of crystallisation. These crystalline chelates did not melt on heating to 300°. The $Fe^{++}$ chelate alone was soluble in chloroform.

Attempts to prepare pure chelates of hexachlorophene were unsuccessful. Using 70 per cent ethanol solution in a similar manner to that used for preparing thiobisdichlorphenol chelates, only amorphous materials were obtained which melted over the range 154° to 210° and probably consisted of hexachlorophene (m.p. 164°) and metal chelate. Hexachlorophene M/100 in dilute alkali containing the stoichiometric amount of NaOH for neutralisation of the two hydroxyl groups was allowed to react with 0·5, 1 and 2 mole equivalents of M/100 $CuSO_4$ solution. With 0·5 mole equivalent a deep brown solution was formed but with 1 or 2 equivalents the brown colour formed initially was followed by the separation of a brown precipitate. The yield of precipitate and the finding of almost one equivalent of $Cu^{++}$ in the filtrate using 2 mole equivalents of $CuSO_4$, indicates that a 1:1 complex could be formed.

This complex was unstable on shaking with chloroform. The brown colour was discharged due to apparent breakdown and extraction of the hexachlorophene into the chloroform layer.

## EXPERIMENTAL

### *Colorimetric Investigations*

*Combining ratios of thiobisdichlorphenol chelates. Solutions.* 0·039M thiobisdichlorphenol was prepared by dissolving the dry material (m.p. 186°) in 85 per cent aqueous ethanol. 0·039M solutions of A.R. grade metal salts were prepared in distilled water at the time of measurement. Ferrous sulphate was used for the $Fe^{++}$ chelate, ferric alum for the $Fe^{+++}$ chelate and copper sulphate for the $Cu^{++}$ chelate. Various ratios of metal salt to chelating agent were used, the total volume being 4 ml. for iron salts and 8 ml. for copper. The thiobisdichlorphenol was added to a 100 ml. volumetric flask from a pipette and 70 ml. of 95 per cent ethanol added. This was followed by the metal salt and the solution then made up to volume with distilled water. The pH for maximum colour development was determined by adjustment of a 1 : 1 mixture of chelating substance and metal salt, made up as above, with 5 per cent NaOH solution, usually 0·1 to 0·3 ml. Absorptiometric measurements were in a 1 cm. glass cell using a 520 m$\mu$ filter. The pH was measured with a Beckman pH meter.

*Potentiometric titrations.* Hexachlorophene (m.p. 164°) and thiobisdichlorphenol (m.p. 186°) were weighed directly and dissolved in 70 ml. of 95 per cent ethanol. The metal salt (A.R. grade) was either weighed directly and dissolved in distilled water (30 ml.) or made up in aqueous solution and an aliquot taken. The mixture was titrated with 0·1N NaOH added from a 10 ml. burette, readings every 0·5 ml. being taken on a pH meter with external glass and palladium junction electrodes. A glass stirrer was used for $Fe^{++}$, $Fe^{+++}$ and $Cu^{++}$ and nitrogen gas for $Co^{++}$, $Mn^{++}$ and $Mg^{++}$. The meter was checked with buffer pH 7 before and after each determination.

*Crystalline chelates. Ferrous chelate.* 25 ml. of a 0·039M ethanolic solution of thiobisdichlorphenol was diluted with ethanol (45 ml.) and 12·5 ml. of freshly prepared aqueous 0·039M ferrous sulphate was added with shaking. Distilled water (18 ml.) was then added and the solution adjusted to pH 6·6 with 10 per cent NaOH. Distilled water (100 ml.) was then added and the mixture allowed to stand in the refrigerator. Purple black needles separated after one day. They were collected and washed with a small volume of cold 50 per cent ethanol. Yield 0·33 g. (88 per cent). Insoluble in water, soluble in alcohol and chloroform.

Analysis. Found, C, 37·0; H, 2·0; Cl, 34·1; S, 7·4 per cent. Calculated for $C_{24}H_8O_4S_2Cl_8Fe{\cdot}H_2O$: C, 36·8; H, 1·28; Cl, 36·3; S, 8·2 per cent.

*Copper chelate.* 25 ml. of ethanolic 0·039M thiobisdichlorphenol, 45 ml. of ethanol and 12·5 ml. of aqueous 0·039M copper sulphate were mixed together and water (18 ml.) added. The mixture was adjusted to pH 7·5 with NaOH. On adding 100 ml. of water light brown micro needles separated. Yield 0·1 g. (25 per cent).

Analysis. Found: C, 33·3; H, 1·77; Cl, 33·7; S, 6·9 per cent. Calculated for $C_{12}H_4Cl_4O_2SCu{\cdot}H_2O$: C, 33·1; H, 1·38; Cl, 32·6; S, 7·3 per cent.

*Copper chelate from aqueous solution.* 25 ml. of 0·039M thiobisdichlorphenol (in 0·1N NaOH) was mixed with 25 ml. of freshly prepared copper sulphate 0·039M. The brown precipitate which formed was collected at the pump, washed with water and dried in a dessicator. Yield, 0·34 g. (80 per cent).

Analysis. Found, C, 31·3; H, 1·54 per cent.

*Cobalt chelate.* Thiobisdichlorphenol (0·178 g.) was dissolved in 70 ml. of 95 per cent ethanol and 30 ml. of a solution of cobalt chloride (0·496 g.) in 250 ml. of water was added. The solution was mixed with a stream of nitrogen and adjusted to pH 7·7 with 0·1N NaOH. After 5 minutes mixing the semi-crystalline material was collected at the pump, washed with a small volume of 50 per cent ethanol and dried in a dessicator. Yield 0·1 g. (53 per cent). Insoluble in water and chloroform.

Analysis. Found, C, 32·2; H, 3·64; S, 6·9 per cent. Calculated for $C_{24}H_8S_2O_4Cl_8Co{\cdot}7H_2O$: C, 32·6; H, 2·47; S, 7·1 per cent. The compound on drying overnight at 110° lost weight corresponding to 7 moles of water. It could be crystallised from ethanol as almost colourless needles.

*Methylene ether of hexachlorophene.* Hexachlorophene (4·1 g.) and methylene bromide (2 ml.) were dissolved in dry butanol (20 ml.) and added to dry butanol (50 ml.) in which sodium (0·46 g.) had been dissolved. The solution was refluxed for 24 hours. Two further portions of methylene bromide (1 ml. each) were added to the refluxing solution after 16 and 20 hours. On cooling, crystals were deposited which were collected on the pump and washed with water. Yield 2·25 g. (54 per cent). The compound crystallised from butanol in white needles, m.p. 212°.

Analysis. Found, C, 40·5; H, 1·9; Cl, 50·6 per cent. $C_{14}H_6O_2Cl_6$ requires C, 40·2; H, 1·4; Cl, 50·7 per cent.

*Methylene ether of thiobisdichlorphenol.* This was prepared in a similar method as described above from thiobisdichlorphenol (3·56 g.) and methylene bromide. After 13 hours refluxing the product was collected. Yield 1·62 g. (44 per cent). It recrystallised from butanol as white silky needles, m.p. 188°.

Analysis. Found, C, 42·5; H, 2·1 per cent. $C_{13}H_6O_2Cl_4S$ requires C, 42·8; H, 1·6 per cent.

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# THE MODE OF ACTION OF CHLORINATED BISPHENOL ANTIBACTERIALS

## PART II. BIOLOGICAL STUDIES

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*From Johnson and Johnson Pty. Ltd., Sydney, Australia*

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2:2′-Thiobis(4:6-dichlorphenol) has been found to be bactericidal against *Staphylococcus aureus* in distilled water at a dilution of one part per million. In contrast to oxine, it is not dependent on the presence of trace elements in the medium for activity. The bactericidal concentration was the same against *Staph. aureus* in nutrient broth which was rendered deficient in metal ions. The crystalline copper and iron chelates of the chelating agent were found to show the same bacteriostatic activity as the agent itself against *Staph. aureus*. However dilution of solutions of these chelates with a large volume of water was found to cause some breakdown to a mixture of agent and metal chelate. Thiobisdichlorphenol and hexachlorophene are suppressed in their action on *Staph. aureus* by ferrous ions, but not by a number of other cations tested. In this, they parallel the behaviour of the tetracycline group of antibiotics. The suggestion has been made that thiobisdichlorphenol and hexachlorophene owe their antibacterial action to an inhibition of certain metal requiring enzyme systems.

THIOBISDICHLORPHENOL and hexachlorophene are able to chelate copper and iron and of the metals studied, thiobisdichlorphenol alone formed chelates with cobalt and manganese[1]. Since both these bisphenols have similar antibacterial properties, a common mode of action is suggested which may be related to their ability to chelate with iron or copper, or their combination.

### EXPERIMENTAL

#### *Determination of Minimum Inhibitory Concentrations of Thiobischlorphenol and its Metal Chelates*

A *Micrococcus pyrogenes* var. *aureus* culture was kindly supplied by the C.S.I.R.O., Division of Food Preservation, Sydney. This culture was obtained from the Central Public Health Laboratory, Colindale, London, and had the strain number 49/1974 and phage pattern 42 D. *B. subtilis* (Marburg strain) was obtained from the Prince Alfred Hospital, Sydney.

Solutions of thiobisdichlorphenol, crystalline Fe chelate and crystalline Cu chelate in sterile polyethylene glycol 400 were diluted in sterile distilled water and aliquots added to tubes of nutrient broth. Inoculation was made with 24-hour broth cultures of the organisms. Results were read after 24 hours incubation at 37°.

#### *Bactericidal Effect of Thiobisdichlorphenol in Double Distilled Water*

Essentially the procedure of Albert, Gibson and Rubbo[2] was followed. All glassware for this experiment was washed, rinsed in distilled water

alcoholic potash, dilute aqua regia and then repeatedly in double glass-distilled water. Cotton plugs were wrapped in Cellophane to prevent dust contamination. A 24-hour plate culture of *Staph. aureus* on nutrient agar was transferred to a centrifuge tube and washed twice with distilled water by decantation after centrifugation. It was then resuspended in double glass distilled water. Ferrous sulphate, copper sulphate and oxine were analytical reagent grade. Thiobisdichlorphenol had m.p. 186°, the solution was made in redistilled ethanol and dilutions were made in double glass-distilled water. Inoculations, held at 37°, were made with 0·1 ml. of the water suspension of *Staph. aureus.* Subcultures (0·1 ml.) were made into Lab-Lemco broth pH 7·0 immediately, and after every hour for 5 hours. Results (Table II) were read after 24 hours incubation at 37°.

*Inhibition in Oxine Treated Broth*

Nutrient broth (pH 7·3) was exhaustively extracted with oxine and chloroform by the method of Rubbo, Albert and Gibson.[3] Glassware was treated by the method described above. Solutions of thiobisdichlorphenol in redistilled ethanol and oxine in sterile double distilled water were diluted in sterile double distilled water and aliquots added to the treated broth and normal broth. 0·1 ml. of an aqueous suspension of double washed *Staph. aureus* was added to each tube. Results (Table III) were read after 24 hours at 37°.

*Effect of Metal Ions on Bacteriostatic Activity of Thiobisdichlorphenol and Hexachlorophene*

The qualitative filter paper disc method described by Weinberg[4] was used. Solutions of thiobisdichlorphenol and hexachlorophene, made in 70 per cent ethanol, were diluted in sterile double distilled water. Aliquots were added to nutrient agar at 50°, the plates poured and inoculated when cold with a suspension of *Staph. aureus* made by diluting one part of a 24-hour culture with four parts of sterile distilled water. Two drops of this suspension were spread over the surface with a glass rod. Filter paper discs (Whatman No. 43 acid washed paper) were cut 1 cm. square and autoclaved. They were then soaked in 0·1 per cent solutions of the metal salts made in sterile water. All the salts were A.R. with the exception of sodium sulphate, which was C.P. grade. Some discs were also soaked in sterile double glass-distilled water. These discs in sterile Petri dishes were then placed in a vacuum dessicator over $P_2O_5$ for 2 hours. After the inoculated surface of the nutrient agar plates had been allowed to dry for half an hour, the discs, which were still moist, were placed aseptically on the surface of the plates. Control plates containing no drug were also included. After incubation for 48 hours at 37° the plates were examined.

## Results

A comparison of bacteriostatic activity of crystalline iron and copper chelates of thiobisdichlorphenol with the agent alone against *Staph.*

*aureus* and *B. subtilis* is shown in Table I. It would appear that the chelates have the same activity as thiobisdichlorphenol against *Staph. aureus*. Against *B. subtilis* the activity of the iron chelate is the same as thiobisdichlorphenol but the copper chelate is less effective. If the chelates were stable at very high dilution the inference could be drawn that thiobisdichlorphenol was active as a metal chelate, formed by combination with metal ions present in the medium. However, it was found

TABLE I

ANTIBACTERIAL ACTIVITY OF THIOBISDICHLORPHENOL AND ITS METAL CHELATES AGAINST *Staph. aureus* AND *B. subtilis* IN NUTRIENT BROTH AT pH 7·3 AND 37°

| Organism | Compound | Concentration p.p.m. | | | | |
|---|---|---|---|---|---|---|
| | | 10 | 2 | 1 | 0·2 | 0 |
| *Staph. aureus* | Thiobisdichlorphenol | — | — | — | + | + |
| | Copper chelate | — | — | — | + | + |
| | Ferrous chelate | — | — | — | + | + |
| *B. subtilis* | Thiobisdichlorphenol | — | — | + | + | + |
| | Copper chelate | — | + | + | + | + |
| | Ferrous chelate | — | — | + | + | + |

that when the crystalline iron and copper chelates, dissolved in polyethylene glycol 400, were diluted with a large volume of water, partial hydrolysis occurred and a mixture of the chelate and agent was precipitated.

The bactericidal activity of thiobisdichlorphenol in the presence and absence of metals was then examined. *Staph. aureus* when suspended in distilled water remains viable for 24 hours as can be demonstrated by plating out. Albert and others[2,3] found that oxine, at concentrations at which it was strongly bactericidal in broth, was without effect on

TABLE II

EFFECT OF THIOBISDICHLORPHENOL IN ABSENCE AND PRESENCE OF METAL SALTS ON VIABILITY OF *Staph. aureus* IN DISTILLED WATER. SUBCULTURES INTO NUTRIENT BROTH WERE TAKEN AT ZERO TIME AND HOURLY INTERVALS FOR 5 HOURS. TEMP. 37°

| Concentration | | Growth after epxosure (hours) | | | | | |
|---|---|---|---|---|---|---|---|
| Compound | Metal | 0 | 1 | 2 | 3 | 4 | 5 |
| Thiobisdichlorphenol M/356,000 | — | + | — | — | — | — | — |
| ” | $FeSO_4 \cdot 7H_2O$ M/139,000 | + | — | — | — | — | — |
| ” | $CuSO_4 \cdot 5H_2O$ M/125,000 | + | — | — | — | — | — |
| — | $FeSO_4 \cdot 7H_2O$ M/139,000 | + | + | + | + | + | + |
| — | $CuSO_4 \cdot 5H_2O$ M/125,000 | + | — | — | — | — | — |
| Oxine M/100,000 | — | + | + | + | + | + | + |
| Oxine M/100,000 | $FeSO_4 \cdot 7H_2O$ M/139,000 | + | — | — | — | — | — |
| — | — | + | + | + | + | + | + |

*Staph. aureus* in glass distilled water. On adding traces of copper or iron the organism was instantly killed. This type of investigation was made to determine whether thiobisdichlorphenol, like oxine, required the presence of trace elements to be effective. Suspensions of *Staph. aureus* in double glass-distilled water containing the test substances were subcultured into nutrient broth at hourly intervals. Results are given in Table II.

It can be seen that thiobisdichlorphenol is bactericidal in the absence of added trace metals. Oxine used as a control failed to inhibit growth but was potent in the presence of iron. Copper alone was bactericidal, as was found by Albert and his colleagues[2]. The mode of action of thiobisdichlorphenol is not then associated with chelation of metal present in the medium as is the case with oxine, which is believed to enter the cell as a 1 : 2 chelate with iron. It was then decided to determine whether thiobisdichlorphenol possessed any increase in activity in a medium rendered deficient in trace metals by extraction with oxine. This was done using the method of Waring and Wekman[5] as adapted by Albert and others[3]. Results are given in Table III.

TABLE III

BACTERIOSTATIC EFFECT OF THIOBISDICHLORPHENOL ON *Staph. aureus* IN NORMAL AND METAL DEFICIENT BROTH. OXINE USED AS A CONTROL. GROWTH OF *Staph. aureus* AT 37° AND pH 7·3

| Medium | Thiobisdichlorphenol concentration p.p.m. | | | | | | Control |
|---|---|---|---|---|---|---|---|
| | 2 | 1 | 0·4 | 0·2 | 0·1 | 0·05 | |
| Oxine treated broth | − | − | + | + | + | + | + |
| Normal broth | − | − | + | + | + | + | + |

Oxine at 1·45 p.p.m. in oxine treated broth gave +ve growth.

Since oxine at 1·45 p.p.m. failed to inhibit growth in oxine treated broth, this demonstrates that the medium was rendered practically metal free by the treatment used. Failure of thiobisdichlorphenol to exhibit any increase in bacteriostatic power in this medium coupled with the fact that it retains its activity in distilled water, means that either the mode of action is not associated with chelation or that, if it is, it may be connected with inhibition of an enzyme by combination with a metal normally required by, and perhaps attached to, the enzyme. Gould and others[6] have found that certain bisphenols, and in particular hexachlorophene, effectively inhibit heart, kidney and liver succinoxidase systems of animal tissues. In another paper[7] they reported that hexachlorophene, G5 [2:2-'methylene bis(4:6-dichlorphenol)] and G11-S [2:2-'thiobis(3:4:6-trichlorphenol)], all at very low concentration, effectively inhibited the glucose, lactic and succinic dehydrogenases as well as the cytochrome oxidase systems of *B. subtilis* and *E. coli.*

Further investigation of the influence of metal ions on the antibacterial activity of thiobisdichlorphenol and hexachlorophene has revealed a close analogy between these compounds and the tetracycline group of antibiotics. The latter group have an avidity for metal ions. Oxytetracycline and chlortetracycline[8] have been found to chelate with $Fe^{++(+)}$, $Cu^{++}$, $Ni^{++}$, $Co^{++}$, $Zn^{++}$ and $Mn^{++}$. The structures of these compounds possess phenolic, enolic and ketonic sites at which chelation can take place[9]. They have been found to inhibit respiration, fatty acid oxidation, oxidative phosphorylation and adaptive enzyme formation[10]. In general, the antimicrobial action of the tetracycline is unaffected by the majority of the ions with which they form chelates. However, a few multivalent ions are capable of suppressing the action of the drugs. The most active

of these ions are $Fe^{++}$ and $Mg^{++}$, which are the ions most often essential for various microbial metal-requiring enzymatic systems[11]. Weinberg[10] has suggested that this reversal or suppression of the activity of the drug by specific metal ions is due to either the metal ions supplying the essential cations required by an enzyme, or by their combination with the drug to remove them from the enzymatic sites.

TABLE IV

EFFECT OF VARIOUS SALTS ON THE BACTERIOSTATIC PROPERTIES OF THIOBISDICHLORPHENOL AND HEXACHLOROPHENE AGAINST *Staph. aureus*. PAPER DISCS SOAKED IN 0·1 PER CENT SOLUTIONS OF THE VARIOUS METAL SALTS WERE PLACED ON INOCULATED AGAR PLATES CONTAINING VARIOUS CONCENTRATIONS OF THE BISPHENOLS

| Medium | Drug | Disc soaked in | Concentration drug p.p.m. | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 | 0·5 | 1 | 2 | 4 | 10 |
| Nutrient agar | Thiobisdichlorphenol | $MgSO_4$ (1) | C | C | D | + | – | – |
| | | $MnSO_4$ (2) | C | C | D | + | – | – |
| | | $CuSO_4$ (3) | C | C | D | – | – | – |
| | | $CoCl_2$ (4) | C | C | D | + | – | – |
| | | $FeSO_4$ (5) | C | C | D | + + | + | – |
| | | $FeNH_4(SO_4)_2$ (6) | C | C | D | + + | + | – |
| | | $Na_2SO_4$ (7) | C | C | D | + | – | – |
| | | Distilled water (8) | C | C | D | + | – | – |
| Nutrient agar | Hexachlorophene | Salts (1)–(4) | C | C | D | + | – | – |
| | | $FeSO_4$ (5) | C | C | D | + | + | – |
| | | $FeNH_4(SO_4)_2$ (6) | C | C | D | + | – | – |
| | | $Na_2SO_4$ (7) | C | C | D | + | – | – |
| | | Distilled water (8) | C | C | D | + | – | – |

C, growth throughout plate. D, growth around disc greater than growth on rest of plate. +, growth around disc, none on rest of plate.

The suppressive ability of a number of metallic ions was tested against various concentrations of hexachlorophene and thiobisdichlorphenol in nutrient agar using *Staph. aureus* as the test organism. Weinberg's[4] technique was used, which makes use of filter paper discs soaked in a 0·1 per cent solution of the ion to be tested. These are then placed on the inoculated surface of the agar. Results, reported in Table IV, were obtained after 48 hours incubation at 37°.

## DISCUSSION

From Table IV it is seen that both $Fe^{++}$ and $Fe^{+++}$ suppress the activity of thiobisdichlorphenol against *Staph. aureus* whilst with hexachlorophene $Fe^{++}$ alone causes suppression at drug concentrations of 1 in 250,000. No other ions caused suppression of antibacterial power at this level. Growth around the discs at 1 in 500,000 was evidently caused by a dilution effect since it occurred with the disc soaked in distilled water. All discs were still moist when placed on the plates.

$Fe^{++(+)}$ has been found to be capable of suppressing the antibacterial action of the tetracyclines against every organism tested[11]. Other cations such as Mg, Mn and Ca are also capable of suppression, but whereas Fe is effective against each genus, Mg is only effective with half the genera and Mn and Ca with only an occassional genus[11]. Evidence then exists for postulating a common mode of action of the most active chlorinated bisphenols and the tetracyclines. The rigidity of the molecular

structures of thiobisdichlorphenols and hexachlorophene as described in Part I bear a formal resemblance to the structure of the tetracyclines. Iron is chelated by all these compounds and since this is the only metal capable of suppression of the activity of *every* compound, inhibition of an iron containing enzyme system is probably a common mode of action of both groups of antibacterials against certain bacteria.

Phenols in general are known to be effective in the unionised state. This property is responsible for the loss in activity in alkaline conditions. Hexachlorophene and thiobisdichlorphenol are the only phenolic antibacterials able to retain the greater part of their activity in mildly alkaline conditions, e.g., in soap solution[12,13]. An explanation for this retention of activity has been offered in terms of the poor ionisation of the second hydroxyl group in mildly alkaline conditions. However, it can be seen from Fig. 3 (Part I) that the iron chelate of thiobisdichlorphenol with maximum stability at pH 7·5, is comparatively stable up to a pH of about 9. This property can then account for the retention of activity in alkaline conditions, utilising the chelation ability as the mode of action of these compounds.

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# ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

## CHEMISTRY

### ALKALOIDS

**Morphine, Codeine and Thebaine, Origin of the Methyl Groups in.** A. R. Battersby and B. J. T. Harper. (*Chem. Ind.*, 1958, 365.) Methyl-$^{14}$C-L-methionine and $^{14}$C-sodium formate have been fed separately to growing *Papaver somniferum* plants. Separation of the alkaloids found by counter-current distribution showed that in both experiments morphine, codeine and thebaine were radioactive. Cleavage of *O*- and *N*-methyl groups separately from each alkaloid (with hydriodic acid), fixation of the liberated methyl iodide as tetramethylammonium reineckate, and determination of the respective activities showed that the methyl groups of morphine, codeine and thebaine accounted for between 55 and 78 per cent of the total activity of the molecules in the methionine experiment. Results from the formate experiment were less conclusive on account of the low specific activities of the alkaloids. The results show, however, that both *O*- and *N*-methyl groups of the opium alkaloids can be derived from methionine, and less efficiently from formate, and are in accord with similar studies using them as precursors for the alkaloids hordenine, protopine, ricinine, hyoscyamine and nicotine. J. B. S.

### ANALYTICAL

**Adrenaline and Related Compounds, Determination of, on Paper Chromatograms.** G. L. Ellman. (*Nature, Lond.*, 1958, **181**, 768.) The most sensitive chemical method for the estimation of adrenaline is the reaction with ethylenediamine. This reaction yields a number of products, some of which are fluorescent and which can be used for quantitative measurement. Noradrenaline produces a fluorescence of different colour so that mixtures of the two substances can be determined. The number of products formed in this reaction was determined by chromatographing the products formed by the interaction of the diamine and various catechols. This was done on paper using as solvent 5 per cent ammonium hydroxide and *n*-propanol (9:1). It was found that adrenaline yields some products different from those given by noradrenaline but under these conditions the products from catechol, noradrenaline, hydroxytyramine and epinine migrate similarly. The addition of ferricyanide or iodide to the ethylenediamine does not change the qualitative nature of the fluorescent pigments but does increase the amounts formed. Details of the method are then given. With such a method 0·03 $\mu$g. of adrenaline can be detected. M. M.

**Solanaceous Alkaloids in Pharmaceutical Preparations, Colorimetric Estimation of.** I. Nir-Grosfeld and E. Weissenberg. (*Drug. Standards*, 1957, **95**, 180.) Two colorimetric procedures are described, each of which is claimed to assay as little as 20 $\mu$g. of alkaloid. In both methods the alkaloids are first converted to the nitro derivatives. In the first method, (I) based on the Vitali reaction, after extraction with chloroform as described in the U.S.P. XV, an aliquot containing 0·25 to 1 mg. of alkaloid is evaporated to dryness on a water bath and nitric acid added. After heating until all the fumes have been driven off, the residue is dried at 105° for 15 minutes and cooled to room temperature. It is then dissolved in acetone and quantitatively transferred to a 25 ml. flask

and made up to the mark with acetone. To 5 ml. of this solution is added, with shaking, 2 ml. of *iso*propylamine and 0·1 ml. of 0·1 per cent methanolic potassium hydroxide. Exactly 1 minute after, readings are taken in a photometer at 540 m$\mu$. Comparison is made with a standard curve prepared using pure atropine sulphate. In method (II), following extraction and nitration as for I, the residue is dissolved in 10 ml. of 50 per cent ethanol. To this is added 2·5 ml. of 10 per cent hydrochloric acid and 0·1 g. zinc dust, and the mixture heated on a water bath for 10 minutes. The mixture is then cooled to room temperature, filtered into a 25 ml. flask, washing the residue. One ml. of 1 per cent sodium nitrite is added, and after mixing, the contents are allowed to stand for 10 minutes. Excess nitrite is destroyed by the addition of 1 ml. of 2·5 per cent solution of ammonium sulphamate. Ten minutes later 1 ml. of 1 per cent aqueous solution of *N*-naphthylethylenediamine dihydrochloride is added, and the mixture made up to the mark with distilled water. After colour development for 30 minutes, readings are made at 550 m$\mu$ and compared with a standard curve as in I. A large number of different pharmaceutical preparations containing solanaceous alkaloids were assayed and checked by these methods, and results obtained were found to be both reproducible and satisfactory. J. R. F.

**Strychnine, Colour Reactions for.** M. Pečar. (*Acta pharm. Jug.*, 1957, **7**, 75.) When a small quantity of strychnine base is heated with sodium selenite and hydrochloric acid (37 per cent HCl) an intense red colour develops. On prolonged heating the colour changes to golden yellow. On cooling, a fine red precipitate forms, which is readily dispersed to give a liquid which appears light orange-red in reflected light, and light yellow by transmitted light. When 0·5 ml. of a solution containing strychnine is acidified with a drop of hydrochloric acid and mixed with a drop of a 10 per cent solution of potassium dichromate, the appearance depends upon the quantity of strychnine present, and with relatively low concentrations of the alkaloid an approximate assay may be carried out by measuring the intensity of the colour produced.

G. B.

## PHARMACY

**Alginate Mucilages, Investigations of their Preparation and Rheological Behaviour.** R. Bolliger and K. Münzel. (*Pharm. Acta Helvet.*, 1958, **33**, 141.) The authors show that alginate solutions are pseudoplastic liquids, the viscosity of which decreases as the shearing stress increases. The pseudoplastic nature of the solutions is proved by the fact that the shearing stress is not proportional to shearing speed, as it would be in the case of an ideal liquid. It is also shown that the viscosity falls very rapidly with increase of temperature. For example, the viscosity in one case was only about 10 per cent at 90° of what it was at 10°. At temperatures of 70° or over there is evidence of some depolymerisation since the final viscosity is less. 70° is thus the maximum temperature recommended for the preparation of mucilages. Thus while it is possible to prepare mucilages by direct solution in water with vigorous stirring, it is best to rub down the alginate with sufficient ethanol or glycerol (2–4 per cent of the final volume), and then to add the water slowly with constant stirring. Such mucilages reach their maximum quasiviscosity within 1 hour, but the value soon begins to fall to a fairly constant value after 24 hours, although the viscosity must still be considered as very unstable. D. B. C.

## PHARMACOGNOSY

**Aloe Drugs, Paper Chromatographic Investigation of.** W. Awe, H. Auterhoff and C. L. Wachsmuth-Melm. (*Arzneimitt.-Forsch.*, 1958, **8**, 243.) The paper chromatography of various species of *Aloes*, especially that of Cape aloes was reviewed and investigated. The process was carried out using paper 4 cm. × 50 cm. (Schleicher and Schüll 2043b Mgl.) and a butanol-glacial acetic acid-water (40 : 10 : 50) as solvent. One drop of a 1 per cent methanolic solution of the drug was used. The spots were viewed in ultra-violet light before and after development with 0·5N KOH solution. This alkali treatment revealed other spots. In Cape aloes the following spots were observed: $R_F$ 0·5, an unidentified anthracene derivative possibly containing 1 : 8-dihydroxyanthraquinone structure; $R_F$ 0·65, Cape aloin; $R_F$ 0·81, *p*-cumaric acid and resin; $R_F$ 0·88, aloe-emodin. D. B. C.

## PHARMACOLOGY AND THERAPEUTICS

**Antitussive Agents, Pharmacological Studies on New Synthetic.** C. I. Chappel, M.-G. P. Stegen and G. A. Grant. (*Canada J. Biochem. Physiol.*, 1958, **36**, 475.) Three basic alkoxyalkyl esters of phenothiazine-10-carboxylic acid were synthesised and tested for antitussive activity, antispasmodic activity, local anaesthetic activity and acute toxicity. Antitussive activity was studied in lightly anaesthetised cats, "coughing" being induced by electrical stimulation of the superior laryngeal nerve and recorded on a revolving drum by means of a writing lever attached to the abdominal muscles just below the sternum. All three compounds possessed antitussive activity in the range of activity of codeine. The most potent antitussive agent was dimethylaminoethoxyethyl phenothiazine-10-carboxylate. It was shown to possess a moderate local anaesthetic action but only slight antispasmodic activity and low acute toxicity. Further studies on this compound showed it to be devoid of central depressant or analgesic properties and no evidence of chronic toxicity in the rat or dog was obtained. As a result of these findings the dimethyl derivative was chosen for clinical trial. W. C. B.

**Cutaneous Absorption from Ointment Bases, Study of Pharmacological Methods for the Evaluation of.** G. Valette and M. Huerre. (*Ann. pharm. franç.*, 1957, **15**, 601.) Physostigmine was incorporated into a series of ointment bases, and the speed of absorption by the skin of rats was determined by measurement of the increase in amplitude of contractions of the muscle on electrical stimulation of the sciatic nerve. Further experiments were carried out using leptazol, and assessing absorption by measuring the time interval before the occurrence of convulsions in mice. Absorption of testosterone was assessed by observations on the development of the comb in chicks. There were some discrepancies between the results by the three methods, but in all cases soft paraffin, lanolin-soft paraffin, and water-in-oil emulsion bases (emulsified with triethanolamine stearate) were ineffective in promoting absorption. Lard assisted the penetration of testosterone, but not of physostigmine or leptazol. Cold cream basis increased penetration to some extent, but an anhydrous basis of *p*-cymene with polyethylene favoured penetration more than any other type of base investigated. G. B.

**5-Hydroxytryptamine, Histamine, Dextran, Eggwhite and Compound 48/80, Inhibition by Various Substances of Oedema Formation in the Rat Induced by.** J. R. Parratt and G. B. West. (*Brit. J. Pharmacol.*, 1958, **13**, 65.) Dextran and eggwhite in the rat cause oedema by the release of 5-hydroxytryptamine. Drugs which deplete the tissues of 5-hydroxytryptamine (5-HT) or block its actions reduce or prevent the oedema. Oedema-producing substances were injected into the rat hind paw and the oedema assessed from the swelling and blueing of the paw, after an intravenous injection of Evan's blue. Of the 5-HT antagonists, 2-bromolysergic acid diethylamide prevented oedema formation by 5-HT, eggwhite and compound 48/80 but not histamine. Of the antihistamines, mepyramine and phenindamine were weak in inhibiting the oedema formation by most agents. Thenalidine was most efficient against dextran and eggwhite. The antimetabolites of 5-HT, 5-methoxy-2-methyl-tryptamine and 1-benzyl-5-methoxy-2-methyltryptamine did not markedly influence oedema production. Adrenaline was a potent inhibitor of dextran and eggwhite oedema and this was not due to vasoconstriction since noradrenaline was much less active. Of the phenothiazine derivatives the most active was the new compound methotrimeprazine which has potent antihistamine and anti-5-HT actions. This compound was the most active antagonist of the locally induced anaphylactoid reactions so far tested. The results of these experiments indicate that both histamine and 5-HT play rôles in increasing capillary permeability in the rat. G. F. S.

**5-Hydroxytryptophan, Pharmacological Studies of.** D. F. Bogdanski, H. Weissbach and S. Udenfriend. (*J. Pharmacol.*, 1958, **122**, 182.) In previous experiments it has been shown that 5-hydroxytryptophan (5-HTP), when given to animals, rapidly penetrates into most tissues and is converted to serotonin wherever the enzyme 5-HTP decarboxylase is present. In dogs it was found possible to raise the brain level of serotonin to 10 times normal by the administration of its precursor. The studies reported here consist of a general survey of the pharmacological actions of 5-HTP. It was found that the administration of 5-HTP to dogs, cats, rabbits, rats and mice produced somatic, autonomic and behavioural effects which resembled those of lysergic acid diethylamide. Those effects were associated with a rise in the serotonin levels in the blood, the central nervous system and the peripheral tissues. In dogs and cats low doses of 5-HTP decreased spontaneous activity. Larger doses caused excitement and disorientation accompanied by depressant effects on certain reflexes, motor control and sensory functions. Behavioural effects included disorientation and fear in dogs, and sham rage in cats. Rodents showed excitement and increased spontaneous activity. Large doses produced excitement followed by depression in all species. 5-HTP also produced mydriasis, piloerection, tachycardia, salivation, lacrimation, tachypnoea, retching, vomiting and increased intestinal motility. There was a marked increase in the serotonin concentration in the rat uterus. Much of this serotonin was found to be pharmacologically inactive. The possible functional significance of serotonin in the brain is discussed and it is suggested that 5-HTP may prove a useful tool in studying the function of serotonin. As a means of administering serotonin, 5-HTP offers a number of advantages over injection of the amine. Unlike serotonin, 5-HTP readily penetrates the blood-brain barrier and forms serotonin within the central nervous system. 5-HTP is converted to serotonin at sites where it is normally formed so that some of its effects may more nearly reflect the physiological functions of the amine. 5-HTP permits the maintenance of relatively high levels of tissue serotonin for long periods of time, while serotonin itself is rapidly destroyed when administered exogenously. M. M.

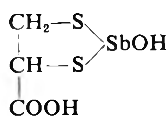
**Mecamylamine, Gastrointestinal Secretion and Absorption of.** E. J. Zawoiski, J. E. Baer, L. W. Braunschweig, S. F. Paulson, A. Shermer and K. H. Beyer. (*J. Pharmacol.*, 1958, **122**, 442.) Mecamylamine, after intravenous or oral administration, was secreted by the gastric mucosa of unanaesthetised Heidenhain pouch dogs with total antrum resections. A definite relationship existed between the amount of mecamylamine secreted and the acidity of the gastric secretions. In the presence of highly acidic gastric juice induced by stimulation with sodium acetate, histamine or acetyl-$\beta$-methylcholine, the secretion of mecamylamine was greatly enhanced. Simultaneous parotid secretion studies did not reveal the presence of mecamylamine in salivary collections and the suggestion is made that the alkalinity of the saliva might account for this. It was found that little, if any, mecamylamine was absorbed by the gastric mucosa in experiments in which mecamylamine was instilled directly into the gastric pouches. In a further series of experiments carried out on anaesthetised dogs, mecamylamine was injected into the lumen of the small intestine. At various time intervals after injection, intestinal loops were excised and the amount of mecamylamine remaining in them was determined. Plasma drug levels were determined simultaneously and were consistent with the interpretation that the decrease in recovery from the lumen was the result of absorption from the intestinal loops. At no time during these tests was any mecamylamine secreted into the small intestine of the dogs. The results demonstrate that there is a definite basis for a gastrointestinal cyclisation of mecamylamine which is favourable to its over-all physiological economy.

W. C. B.

**Rauwolfia A—An Alkaloid from *Rauwolfia verticillata* (Lour.) Baill, Pharmacological Studies on.** L. Chi-chiang and C. Wei-chow. (*Acta Physiol. Sinica.*, 1957, **21**, 283.) Rauwolfia A, isolated recently by Chou from *R. verticillata* collected from Kwangtung province, was investigated for its effects on blood pressure, respiration, heart and acute toxicity. The alkaloid produced a hypotensive effect on anaesthetised rabbits and cats. In cats anaesthetised with pentobarbitone intravenous injections of 2 mg./kg. produced a prompt fall of arterial pressure and an average reduction of 27 per cent. After 1½–2 hours the blood pressure recovered gradually. Repeated administrations revealed no significant tachyphylaxis. There was bradycardia and, in most cases, slight depression of the respiratory amplitude during the early phase of pressure falling, but no influence on the respiratory rate. Rauwolfia A inhibited the pressor responses to electrical stimulation of the afferent vagi and sciatic nerves and also to the carotid occlusion pressor reflex. After its administration, acute carotid sinus neurotomy (after vagotomy) did not alter the blood pressure. The hypotensive effect was not eliminated by atropinisation or vagotomy. It caused inhibition and reversal of the pressor response to injected adrenaline. A larger dose of rauwolfia A inhibited the direct vasoconstriction caused by stimulation of splanchnic nerves, and reversed the subsequent adrenergic pressor effect. Thus, it may be regarded as having both a central site of action and adrenolytic effect. It produced a slowing of heart beat on the perfusion of isolated toads' hearts. In 1:5,000 dilution, A–V block occurred. It caused increase of the output in the concentration of 1:10,000 and 1:25,000. Although the minute output was reduced gradually at the lower dilutions, no significant change in stroke volume for each beat was recorded. In mice the acute LD50 after intraperitoneal injection of rauwolfia A was found to be 0·34 g./kg. It produced severe emesis but no respiratory failure or loss of equilibrium in pigeons at the dose of 20 to 40 mg./kg. given intravenously.

**Serotonin, Displacement of, from Tissues by a Specific Antimetabolite.** D. W. Woolley and P. M. Edelman. (*Science*, 1958, **127**, 281.) The aim of this work is to determine whether BAS (1-benzyl-2-methyl-5-methoxytryptamine), the benzyl analogue of serotonin, would displace serotonin in animal tissues. Rabbits were given 15 mg./kg. of BAS daily for 3 days. The serotonin content of the blood platelets was then assayed, both colorimetrically and pharmacologically on the isolated uterus of the rat. It was found that this treatment with BAS lowered the serotonin content of the platelets by about 50 per cent. Measurements were also made on the effect of BAS administration on the serotonin content of the stomach and the small intestine of mice. In these tissues there was only a slight reduction in the serotonin content. Measurements on the urine of mice treated with BAS showed that excretion of 5-hydroxyindoleacetic acid was not increased but that the urinary excretion of serotonin was markedly increased. Apparently the BAS inhibited the action of amine oxidase and consequently the displaced serotonin was excreted largely unchanged. The finding that there is a displacement of serotonin from tissues by a specific antimetabolite may have some bearing on the mode of action of reserpine. It may be that some of the pharmacological actions of reserpine arise from this antiserotonin property. The ability of reserpine to displace serotonin from tissues is a type of action possessed by specific antimetabolites, such as BAS. M. M.

**Schistosomiasis japonica, Activity of Compounds with Dithiadihydrostibiol Structure against, in Mice.** T. Yea-lin, C. Chiao-chen, C. Chih-chiang, L. Yu-i and T. Kuang-sheng. (*Acta physiol. Sinica*, 1956, **20**, 125.) A series of 8 new compounds with the dithia-dihydrostibiol structure has been compared for therapeutic activity against schistosomiasis japonica in white mice. Each drug, thoroughly mixed with the finely powdered food, was fed to healthy mice for 14 days, and the LD10 and LD50 were evaluated. At the dosages of LD10 and LD50 each drug was fed to treat diseased mice (beginning on the 36th day after percutaneous infection on the abdomen with 40 cercariae per mouse) for 14 days. After a holding period of another 14 days the mice were killed. Both portal system and liver were examined for worms. Basing on the average number of worms remained in each mouse, the therapeutic effects of compounds I and II out of the 8 compounds were better than that of tartar emetic administered orally.

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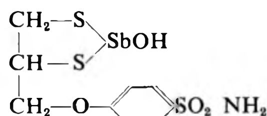


(II)

**Valyl-Oxytocin: Effect on Man.** C. N. Smyth. (*Brit. med. J.*, 1958, **1**, 856.) A comparison was made of valyl-oxytocin, an analogue of synthetic oxytocin, with synthetic oxytocin itself in man. As an excitor of contractions valyl-oxytocin is four times as potent as synthetic oxytocin, even though the two substances are equiactive when assayed by the pharmacopoeial method. If, however, valyl-oxytocin is diluted four times so as to be equiactive with oxytocin then it is also practically equiactive in terms of the rise in milk ejection pressure in the rabbit mammary gland and in its action on the cat uterus *in situ*. Synthetic and natural oxytocin give roughly identical results when tested by any of the above methods and it appears that the milk-pressure assay, or that of the cat uterus *in situ*, might with advantage be used in addition to the pharmapoeial methods. Doses of valyl-oxytocin which are equiactive in the human

subject with oxytocin in exciting uterine contractions are not equiactive in effects on blood pressure. Blood pressure is affected only by extremely large doses of oxytocin, 2 units intravenously usually being required to produce a pressure fall lasting about a minute and occurring 2 to 3 minutes from the time of injection. Half a unit of valyl-oxytocin does not produce this effect but 2 units will do so. In this respect, the pharmacopoeial assay of the two substances on chicken blood pressure or isolated rat uterus is parallel to the comparison in man. Valyl-oxytocin was given to 8 parturient patients by intravenous infusion, using concentrations varying from 0·5 to 4 units per litre of 5 per cent dextrose. It produced a good contractile rhythm, no abnormal effects were noticed, and the babies showed no signs attributable to intrauterine anoxia or abnormal pressures. S. L. W.

**Voacamine and Voacorine, Alkaloids of *Voacanga africana* Stapf., Comparative Pharmacological Study of.** M. A. Quevauviller and O. Blanpin. (*Ann. pharm. franç.*, 1957, **15**, 617.) Experiments using various animals (mouse, guinea pig, rabbit, dog, cat) showed that voacorine and voacamine are 100 to 250 times less toxic than digitalis glycosides, and that voacorine resembles digitalis in its action on the heart. Voacamine differs in stimulating cardiac muscle without slowing the rhythm. Voacamine was shown to have a definite hypotensive effect, with some variation in sensitivity between the animal species used but voacorine appeared to have a hypertensive effect. Both alkaloids induced contraction of smooth muscle, voacorine by direct stimulation of the muscle, and voacamine by parasympathomimetic action. G. B.

## APPLIED BACTERIOLOGY

**8-Hydroxyquinoline Derivatives, Antimicrobial Effect of.** N. Diding and S. Ström. (*Pharm. Weekbl.*, 1958, **93**, 201, also *Svensk. farm. Tidskr.*, 1957, **61**, 508.) The 8-hydroxyquinoline derivatives under examination were ground to fine powder, and sterile suspensions containing 200 $\mu$g./ml. in water were prepared. Serial dilutions were made in nutrient broth and Sabouraud broth and inoculated and incubated. In some of the tests a small proportion of tween 80 or benzalkonium chloride was added to indicate the effect of surface-active agents which are included in some tablet formulations. 5-Chloro-7-iodo-8-hydroxyquinoline and 2-methyl-5 : 7-dichloro-8-hydroxyquinoline were active against staphylococci, enterococci and *Candida albicans*. Some synergistic effect due to the surface-active agent was observed in the case of enterococci. 5 : 7-Diiodo-8-hydroxyquinoline was not effective against these organisms. The derivatives were less effective against *Escherichia coli*, and the addition of tween 80 increased the effect of the 5-chloro-7-iodo derivative only. The substances were not effective against *Pseudomonas aeruginosa*, *Salmonella typhi* and *Proteus vulgaris*, although in some experiments the addition of tween 80 again increased the effect of the 5-chloro-7-iodo compound. All three compounds seemed to have an inhibitory effect on the microflora in man. G. B.

**Vitamin $B_{12}$, Microbiological Assay of, in Antibiotic Preparations.** N. A. Diding. (*Pharm. Acta Helvet.*, 1958, **33**, 156.) A 30-fold increase in resistance to tetracycline of a mutant of *E. coli* was achieved by repeated subculturing of the organism in the presence of increasing concentrations of tetracycline. This could be used to assay a preparation containing $B_{12}$ in the presence of 250,000 to 4,000,000 times the concentration of tetracycline. It is thought that this technique will be of value for the assay of other preparations containing antibiotics and vitamins. D. B. C.