REVIEW ARTICLE

SELENIUM ANALOGUES OF BIOLOGICALLY ACTIVE SULPHUR COMPOUNDS

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PREVIOUS reviews relating to selenium have dealt with the chemistry (Bradt and Crowell, 1932; Painter, 1941) or with the toxic properties (Moxon and Rhian, 1943; Trelease and Beath, 1949; Underwood, 1956; and Moxon, 1958), although a recent review (Schultze, 1960) surveyed the biochemical relationships of selenium-containing Factor 3 and Vitamin E in the animal body. This non-toxic activity of seleniferous material was initially discovered when an essential dietary factor for rats was shown to contain selenium in bound form (Schwarz and Foltz, 1957). Earlier reports had shown the limited essential nature of selenium in plants (Trelease and Trelease, 1938) and in micro-organisms (Pinsent, 1954). While interest in selenium compounds as possible therapeutic agents has been increasing in recent years, this demonstration of the prophylactic-therapeutic properties of Factor 3 can be expected to stimulate further research in this field. In view of the medicinal importance of very many sulphur compounds, it seems appropriate to focus attention on the selenium analogues of some of these and to compare their biological activities.

Isosterism and Bio-isosterism

The earlier work in this field has been well reviewed by Schatz (1960) but a short introductory summary is included. Langmuir (1919) introduced and applied the term "isosteric compounds" or "isosteres" to molecules in which the number and arrangement of electrons was the same. Extensions of this theory led ultimately to the concept that, if compounds "fit the broadest definition for isosteres and have the same type of biological activity", or are directly antagonistic, they are "bio-isosteric" (Friedman, 1951). Nitrous oxide and carbon dioxide, termed isosteric by Langmuir, were later shown to be reversibly anaesthetic to a slime mould, and so fit Friedman's definition of bio-isosteres. Fieser and Richardson (1948) used the term "isolog" in preference to "isostere", although isologous compounds need not always be isosteric. The same terminology was used by Mautner and Clayton (1959) in referring to several series of oxygen, sulphur and selenium compounds, which had previously been described by Mautner (1956) as "almost sterically identical". Isosteres need not be bio-isosteres, but simple isosteric replacements often give compounds of interest and value, and the successful results already obtained through isosteric replacement show that this type of variation is useful in modelling new compounds. Isosterism, however, "will not accomplish for molecules what the periodic table has accomplished for the elements, namely correlation of similar behaviour with similar electronic structure". Molecular size and shape must also be considered in determining biological properties.

Bio-isosterism in the group oxygen, sulphur, selenium and tellurium was mentioned by Schatz (1960), but selenium and tellurium derivatives were considered to be only of minor importance. Friedman (1951) noted that sulphur was surprisingly less bio-isosteric with oxygen than might have been expected, probably due to polarity differences, while Mautner (Mautner and Günther, 1960), in recent extensive work on selenium analogues of biologically active sulphur compounds, has repeatedly emphasised the isosteric similarity between sulphur and selenium. He drew attention to the fact that the radius of doubly bound sulphur (0·94 Å) is close to that of doubly bound selenium (1·07 Å) and that sulphur and selenium analogues crystallised in identical forms which differed from the oxygen analogues (Mautner, 1956; Mautner and Kumler, 1956).

Considering now biological reactions, it was suggested that the mechanism for the excretion of selenium from the animal body resembled that for sulphur (Moxon, Schaefer, Lardy, Dubois and Olson, 1940). Thus dogs excrete sulphur compounds in the presence of bromobenzene as p-bromophenylmercapturic acid (I), and similarly, selenium compounds are excreted as "selenomercapturic acid" (McConnell, Kreamer and Roth,

$$Br \cdot C_6H_4 \cdot S \cdot CH_2 \cdot CH \cdot CO_2H$$
$$| $$
$$NH \cdot CO \cdot Me$$

(I)
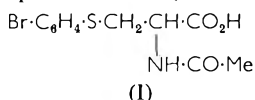
1959). In a different context, Mautner and Günther (1960) have shown that selenopantethine (II, X = Se) can replace pantethine (II, X = S)

$$(HOCH_2 \cdot CMe_2 \cdot CH(OH) \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot X)_2$$

(II)
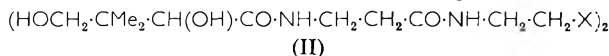
completely on a mole for mole basis in *Lactobacillus helveticus*. The selenium compound, if supplied to the organism preformed, can replace its sulphur analogue in the biogenesis of coenzyme A, which thereafter performs a biological function.

These two random examples illustrate the fact that selenium can react in biological systems as would sulphur, and can in some cases replace it.

*Selenium Analogues of Biologically Active Sulphur Compounds*

Interest in organoselenium compounds as possible therapeutic agents dates back only some twenty years. Matti (1940), stimulated by the antistreptococcal properties of the sulphonamides and sulphones, prepared several selenium compounds with a view to their possible use in the treatment of cancer and leprosy. The compounds prepared were derivatives of diphenyl selenide (III) and of diphenyl diselenide (IV). Some of these were tested in the treatment of leprosy and streptococcal infections.


(III)

R = o- and p-NO$_2$
o- and p-NH$_2$
o- and p-Me·CO·NH

(IV)

R′ = o- and p-NH$_2$
o- and m-Me·CO·NH
p-OH
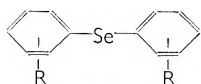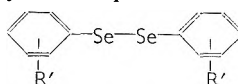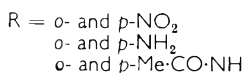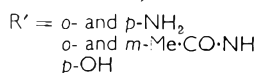
No action was noted against streptococci, while in leprosy, only the simplest derivatives showed favourable activity.

In the same year Painter, Franke and Gortner (1940) investigated some organoselenium compounds with a view to studying selenium compounds in cereals.

Sulphanilamide derivatives containing selenazole (V) and "seleno-hydantoin" (VI) residues were prepared (Roy and Guha, 1945), but no report of their biological properties has been found.

R'—┬——N   OC——N

\\Se/ —NH·SO$_2$·C$_6$H$_4$·NHR   \\Se/ —NH·SO$_2$·C$_6$H$_4$·NH·CO·Me

R' = Me,Ph; R = CO·Me, H

   (V)   (VI)
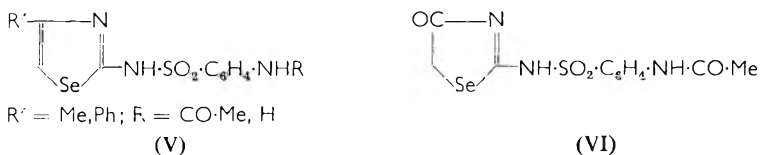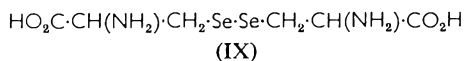
Selenium Amino-acids

Besides these isolated reports, several papers described selenoamino-acid derivatives. A crystalline amino-acid complex containing sulphur and selenium was isolated from *Astragalus pectinatus* (Horn and Jones, 1941) This material analysed as a complex of two parts selenocystathionine (VII, X = Se) and one part cystathionine (VII, X = S). The isolation of such plant products, in a pure form, is extremely difficult since the

$$HO_2C·CH(NH_2)·CH_2·X·CH_2·CH_2·CH(NH_2)·CO_2H$$
(VII)
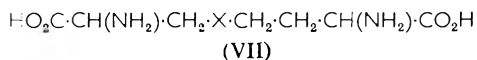
selenium compounds occur in very small proportions in conjunction with analogous sulphur compounds with very closely related properties. This difficulty was further exemplified by the isolation of another seleno-amino-acid from *A. bisulcatus* by the use of ion-exchange- and filter-paper-column techniques (Trelease, Di Somma and Jacobs, 1960). The indications were that this amino-acid was *Se*-methylselenocysteine (VIII, R = Me; X = Se), still not completely separated from S-methyl-cysteine (VIII, R = Me; X = S).
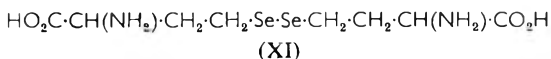
$$R·X·CH_2·CH(NH_2)·CO_2H$$
(VIII)
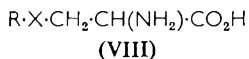
Stekol (1942), in a short note, described the product obtained from the interaction of cysteine hydrochloride and sodium selenite as selenium tetracysteine, which analysed as $Se(C_3H_6NO_2S)_4$, but no structure was given. Fredga (1937) synthesised selenocystine (IX), while ($\pm$)-seleno-cystine (IX) and derivatives of selenocysteine (VIII, X = Se; R = Ph, Ph.CH$_2$) were prepared as part of an investigation of selenium compounds in plants (Painter, 1947a). Painter's approach was to synthesise the

$$HO_2C·CH(NH_2)·CH_2·Se·Se·CH_2·CH(NH_2)·CO_2H$$
(IX)

selenium analogues of sulphur-containing amino-acids and to compare them pharmacologically and chemically with the isolated plant products. This was reasonable, since it was accepted by then that selenium occurred

as a constituent of amino-acid material (Horn and Jones, 1941). This work was extended by Painter (1947b) with the synthesis of the selenium analogues of ($\pm$)-methionine (X) and ($\pm$)-homocystine (XI), since cystine

$$Me \cdot Se \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$$

(X)

$$HO_2C \cdot CH(NH_2) \cdot CH_2 \cdot CH_2 \cdot Se \cdot Se \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CO_2H$$

(XI)
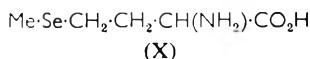
(or cysteine) and methionine carry nearly all the sulphur in cereal proteins. Homocystine, although never identified in plants, was of interest because of its known ability to supply animals with their sulphur-containing amino-acid requirements. In the same year, the synthesis of these two selenium compounds was improved (Klosterman and Painter, 1947). Experiments were later reported by Klug and Petersen (1949) suggesting that selenium tetracysteire (Stekol, 1942) and selenium dicysteine (Painter, 1947a) were really mixtures of cysteine and, probably, selenium dicysteine. This, however, conflicted with other work (Williams and Ravve, 1948), in which the selenium analogue of ($\pm$)-cystine was synthesised by two different routes, to give a compound melting at 215°, compared with 215° (Fredga, 1937) and 222° (Painter, 1947a).
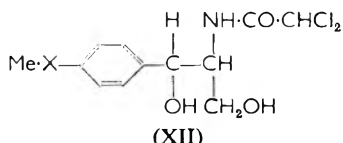
Weisberger, Suhrland and Seifter (1956) reported that selenocystine (IX) was effective in low concentrations in decreasing the incorporation of radioactive L-cystine in leukaemic leucocytes *in vitro*. Diets deficient in the sulphhydryl amino-acid L-cysteine had previously been reported to suppress the growth of malignant tumours in animals. It was not known whether selenocystine also competitively inhibited cystine incorporation in the intact animal, but it was shown (Weisberger and Suhrland, 1956a) that selenocystine decreased the incorporation of L-[^{35}S]cystine by rat murphy lymphosarcoma tumour cells both *in vitro* and *in vivo*, whilst benzylselenocysteine (VIII, X = Se; R = CH₂·Ph) did not. Selenocystine also decreased tumour growth in the intact animal. Under clinical conditions (Weisberger and Suhrland, 1956b), selenocystine had a rapid and striking effect on leukocytes in both chronic and acute leukaemia. The effect was greater on immature than on mature leukocytes and the action took place at the source with a reduction in spleen size, as well as by attack of the leukocytes in circulation. It was of interest to note that selenocystine was effective in patients with acute leukaemia resistant to cortisone, aminopterin or 6-mercaptopurine. A decrease in leukocyte count was also reported in chronic myeloid leukaemia resistant to irradiation, Fowler's solution, urethane and busulphan. One case was quoted where the condition which had become resistant to 6-mercaptopurine became responsive again to 6-mercaptopurine after selenocystine treatment. The mechanism of action was unknown, but selenocystine seemed to have a specific toxicity towards immature leukocytes, since no organic changes, attributable to selenium toxicity, were discernible. Nausea and vomiting, which were often very severe. proved a serious disadvantage with selenocystine and difficulty was found in continuing treatment.
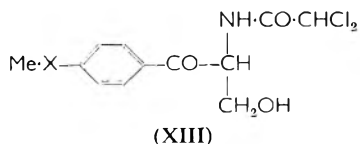
Cowie and Cohen (1957) showed that selenomethionine (X) could completely replace methionine for normal growth of a methionine-requiring mutant of *Escherichia coli*, and that radioactive selenium, from selenite, was incorporated into the bacterial proteins. Selenomethionine was identified in protein hydrolysates of *E. coli* (Tuve and Williams, 1957; 1961) and it was suggested that selenocystine may also be produced by *E. coli* grown in the presence of sodium selenite. Thus, since cystine is an intermediate of methionine production in *E. coli* and since selenomethionine has been identified, it is believed, but so far not proven, that selenocystine may also be produced.

Chloramphenicol Derivatives

The sulphur (XII, X = S) and selenium (XII, X = Se) derivatives of chloramphenicol (XII, X = O) were synthesised (Supniewski, Misztal and Krupinska, 1954) and shown to be strongly antibacterial, with the selenium compound about ten times more active than its sulphur analogue.

$$\text{Me·X} - \underset{}{\bigcirc} - \underset{\underset{\text{OH}}{|}}{\overset{\overset{\text{H}}{|}}{C}} - \underset{\underset{\text{CH}_2\text{OH}}{|}}{\overset{\overset{\text{NH·CO·CHCl}_2}{|}}{CH}}$$
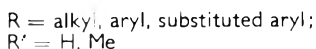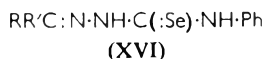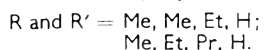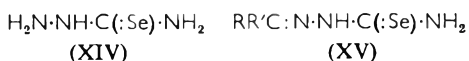
(XII)

Gram-positive were more sensitive than Gram-negative bacteria and both the selenium and sulphur analogues were strongly active against acid-fast bacteria. The toxicity in mice of both compounds was comparable with that of chloramphenicol itself. The lethal doses were 500 mg./kg. weight and the toxic symptoms identical with those of chloramphenicol, while the selenium compound, but not the sulphur compound, decreased respiratory movement, lowered arterial pressure and had a diuretic effect on the cat. The same authors also reported that the ketone derivative of chloramphenicol has strong antifungal activity, but this action was not noted in the sulphur and selenium analogues. No structure was given for these compounds but they are presumed to be as shown in structure (XIII, X = O, S, Se).

$$\text{Me·X} - \underset{}{\bigcirc} - \text{CO} - \underset{\underset{\text{CH}_2\text{OH}}{|}}{\overset{\overset{\text{NH·CO·CHCl}_2}{|}}{CH}}$$

(XIII)

Selenosemicarbazide Derivatives

Selenosemicarbazide (XIV) was isolated in a synthesis of some of its carbonyl derivatives (XV) (Hulls and Renson, 1956a). 4-Phenylselenosemicarbazones (XVI) were also isolated by Huls and Renson (1956b), but no report of their biological activity has been found.

H₂N·NH·C(:Se)·NH₂	RR′C:N·NH·C(:Se)·NH₂	RR′C:N·NH·C(:Se)·NH·Ph
(XIV)	(XV)	(XVI)
	R and R′ = Me, Me, Et, H; Me, Et, Pr, H.	R = alkyl, aryl, substituted aryl; R′ = H, Me

2-Phenylselenosemicarbazide (XVII) and a number of its derivatives (XVIII) were also reported (Mautner and Kumler, 1956), but the synthesis of selenosemicarbazide (XIV) by this method, failed.   As an extension to the chelating theory of action of thiosemicarbazides in tuberculosis it was suggested by Mautner and Kumler (1956) that the selenium

$$Ph \cdot N \cdot C(:Se) \cdot NH_2$$
$$NH_2$$

$$Ph \cdot N \cdot C(:Se) \cdot NH_2$$
$$N:CH \cdot C_6H_4 \cdot R$$
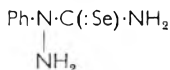
$$R = NO_2, Cl, Br, I, NMe_2, NH \cdot CO \cdot Me, OMe, OH$$

(XVII)   (XVIII)

analogues might have greater chelating ability, since selenium is related to sulphur in the same way as sulphur is to oxygen.   The sulphur compounds showed antitubercular and antifungal activity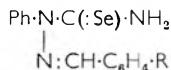, while it was already known that replacement of sulphur by oxygen resulted in complete or partial loss of both types of activity.   When the antifungal properties of 2-phenylselenosemicarbazide (XVII), its selenosemicarbazone derivatives (XVIII), phenylselenourea and their sulphur and oxygen analogues were compared by Mautner, Kumler, Okano and Pratt (1956) against plant and animal pathogens and a saprophytic organism, it was found that the selenium compounds were ten to one thousand times more active on a molar basis than the sulphur compounds, while the oxygen analogues showed negligible activity.   The possibility of released selenium being the active agent was proved to be of little importance.   Finally, it was concluded that selenium compounds were of sufficient activity to warrant further research.

Closely related derivatives of 2-phenylselenosemicarbazide (XVIII, R = NMe$_2$ and (XIX, R = p-Br, p-OEt) were synthesised and tested for

$$Ph \cdot N \cdot C(:Se) \cdot NH_2$$
$$NH \cdot CS \cdot NH \cdot C_6H_4 \cdot R$$
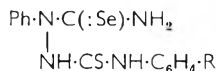
(XIX)

antimicrobial activity (Bednarz, 1957).   These compounds were shown to have a weak effect on staphylococci, *Bacillus subtilis* and *E. coli*, a moderate effect on *Mycobacterium phlei*, *M. smegmatis* and the bacillus of Calmette and Guérin and a very strong effect on *M. tuberculosis*.

Selenopurines and Selenopyrimidines

Using similar methods to those for the preparation of the thio-analogues, selenopurine and selenopyrimidine derivatives have been prepared (Mautner, 1956).   In this way 6-selenopurine (XX), 6-(methylseleno)purine (XXI), 2-selenouracil (XXII), 2,4-diselenouracil (XXIII) and 2-seleno-thymine (XXIV) were isolated.   It had been noted that the most useful
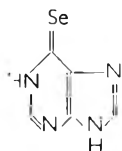
(XX)   (XXI)   (XXII)   (XXIII)   (XXIV)
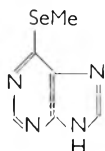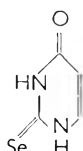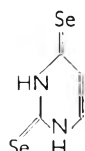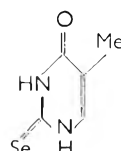
purine and pyrimidine bases were those in which the size of the new atom or group was closely similar to the atom or group replaced. Since the radius of doubly bound selenium is close to that of doubly bound sulphur, the compounds synthesised were sterically almost identical with the sulphur analogues.

The actions of 6-selenopurine and 6-mercaptopurine were compared against Ehrlich ascites tumour systems and *Lactobacillus casei*, as well as against a wide range of micro-organisms (Mautner, 1958). 6-Selenopurine inhibited a 6-mercaptopurine-resistant strain of *L. casei* as efficiently as it did the wild strain. In contrast, mouse leukaemia L-1210, resistant to 6-mercaptopurine, showed full cross-resistance to the selenium compound (Jaffe and Mautner, 1958a). 6-Selenopurine also inhibited the grcwth of a fairly wide range of micro-organisms, being more active than the corresponding sulphur compound, while it was shown that the sulphur and selenium compounds appeared to act by similar mechanisms. Another somewhat more detailed report (Jaffe and Mautner, 1958b) showed that with some tumour systems 6-selenopurine had lower anti-tumour activity and greater host toxicity than equimolar quantities of 6-mercaptopurine and equivalent activity with others. Methylation decreased the activity of both compounds. Since 6-selenopurine was unstable, its effectiveness implied a swift and selective action. The possibility that the decomposition products may be the active species was not supported by the inactivity of the equally unstable 6-(methyl-seleno)purine (XXI) and 2-selenouracil (XXII).

Recently, the synthesis and preliminary biological testing of 6-seleno-guanine (XXV) was reported by Mautner and Jaffe (1961), following from the antimitotic activity of 6-thioguanine, which involved its incor-poration into deoxyribonucleic acid (DNA). Although the mechanism of action of 6-thioguanine was unknown, it was suggested (Mautner and Jaffe, 1961) that charge separation might lead to unusually strong

(XXV)
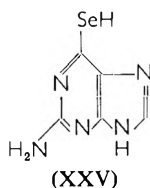
hydrogen bonding with the amino-group of cytosine facing thioguanine in the double helix of DNA and that this could be expected to interfere with its replication. Similar charge separation has been found to be greater in thiocarbamoyl than in carbamoyl compounds, while replace-ment of sulphur by selenium gives even more marked polarisation (Mautner and Clayton, 1959; Mautner, 1956). Since 6-selenopurine shcwed antitumour activity in mice despite its instability (Jaffe and Mautner, 1958b; 1960), 6-selenoguanine was similarly tested, having first been shown to be more stable. Effective growth inhibition of *L. casei* was obtained with 6-selenoguanine with one-tenth the required

thioguanine concentration, but the selenium compound was more toxic to mice than the thio-compound in a single dose, although this position was reversed on repeated administration. *In vivo* testing in mice showed that the two compounds had comparable antitumour activity, while the selenium analogue had an appreciably higher therapeutic index. It was shown that if a tumour showed resistance to 6-mercaptopurine, this extended to thioguanine as well as to selenoguanine.

8-Selenopurines (XXVI), related to known purine antimetabolites,

(XXVI)
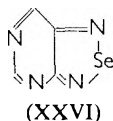
were synthesised as possible antimetabolites for chemotherapeutic studies in cancer (Carr, Sawicki and Ray, 1958). Carcinostatic activity had resulted when the carbon atom in position 8 of guanine was replaced by a nitrogen atom. In this instance selenium was introduced as a more radical change than the replacement of the nitrogen atom, and while the earlier purine derivatives (Mautner, 1956) had an exocyclic selenium atom, the compounds prepared by Carr, Sawicki and Ray (1958) had a heterocyclic selenium atom. The possible mechanism of action was discussed, but no report of the biological testing of these compounds has been noted.

2-Selenobarbiturates

Several 2-selenobarbituric acid derivatives (XXVII) were prepared (Mautner and Clayton, 1959) as part of an investigation of the relative

$R = H, Ph, CH_2 \cdot Ph, C_5H_{11}; R' = H$
$R' = Et; R = Ph$

(XXVII)
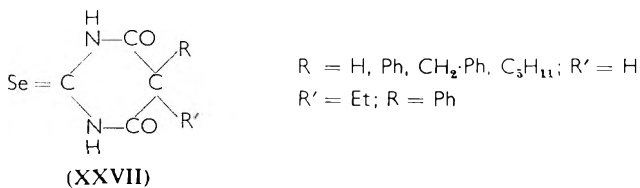
lipid solubilities of oxygen, sulphur and selenium compounds. 6-Seleno-purine (XX) and 2-selenouracil (XXII) were similarly investigated. 6-Selenopurine had slightly greater lipid solubility than the thio-analogue, while 2-selenouracil was less soluble at physiological pH than was 2-thiour-acil. In the case of the barbiturates tested, the sulphur and selenium analogues had very similar lipid solubilities. It was concluded from these results that, for the types of compound tested, replacement of an oxygen by a sulphur atom is an effective method of increasing lipid solubility, with only minor changes in the steric configuration of the molecule. Further replacement of sulphur by the more metallic selenium did not reduce the lipid solubility, and it was concluded that lack of such solubility should not be a major problem in synthesising selenium analogues of biologically active oxygen and sulphur compounds.

Phenoselenazine Derivatives

The selenium analogues of promethazine (XXVIII) and chlorpromazine (XXIX) were isolated by Müller, Buu-Hoï and Rips (1959), and were
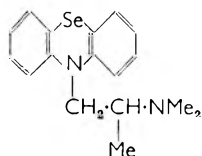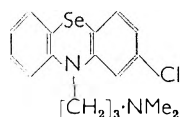
(XXVIII)   (XXIX)

shown to have antihistaminic activity comparable to that of their sulphur analogues.

A number of stable, non-toxic phenoselenazine derivatives (XXX),
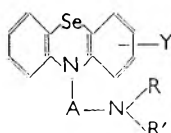
(XXX)

Y = H, halogen, CF₃, lower alkyl or lower alkoxyl group.

A = Divalent, straight or branched alkylene chain of 2–6 carbon atoms.

R,R′ = H, Ph·CH₂, lower alkyl or, with the nitrogen, part of a monocyclic 5- or 6-membered heterocyclic ring.

unsubstituted or substituted in position 10, have been patented (Smith, Kline and French Laboratories, 1959). These compounds were reported to be particularly useful as tranquillisers or mild sedatives. They also showed fungicidal, antibacterial, antihistaminic and anti-emetic properties. Those substituted in position 10, useful mainly as intermediates, also displayed significant antifungal, vermifugal and antibacterial activity.

6-Selenoctic Acid

When Schwarz and Foltz (1957) showed that selenium was an integral part of the biologically active Factor 3, they did not report Factor 3 as a pure chemical entity but described some of its properties, as did Patterson, Milstrey and Stokstad (1957). The suggestion by Bergson (1957) that 6-selenoctic acid (XXXI) might be related to, or even identical with Factor 3, was quickly shown to be incorrect (Schwarz, Foltz and Bergson, 1958).

$$CH_2\text{---}CH_2\text{---}CH \cdot [CH_2]_4 \cdot CO_2H$$
$$Se\text{---}Se$$
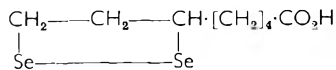
(XXXI)

2-Imino-4-selenazolidones and Selenazolid-2,4-diones

A number of 2-imino-4-selenazolidones (XXXII) and their acid-hydrolysis products, selenazolid-2,4-diones (XXXIII), have been prepared and

R = H, alkyl, Ph.

(XXXII)   (XXXIII)

773
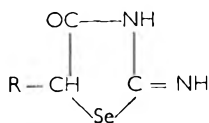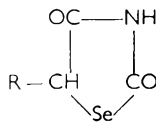
screened for biological and antimicrobial activity. These products were screened for a wide range of activity in view of the diverse activity shown by similar oxygen (Clarke-Lewis, 1958) and sulphur analogues (Brown, 1961). However, no promising activity was shown by the selenium compounds (Comrie, Dingwall and Stenlake, unpublished).

Proposed Metabolic Studies

Pichat, Herbert and Thiers (1961) reported the synthesis of seleno-cystamine (XXXIV), selenohypotaurine (XXXV) and selenotaurine (XXXVI) in a proposed comparison of the metabolism of organic sulphur and selenium compounds.

$$(NH_2 \cdot CH_2 \cdot CH_2 \cdot Se)_2 2HCl \qquad NH_2 \cdot CH_2 \cdot CH_2 \cdot SeO_2H \qquad NH_2 \cdot CH_2 \cdot CH_2 \cdot SeO_3H$$

$$\text{(XXXIV)} \qquad \text{(XXXV)} \qquad \text{(XXXVI)}$$
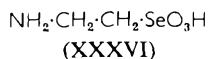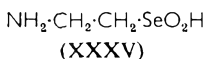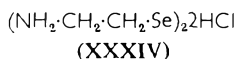
A different approach to the same problem has been taken by Fredga and Lindgren (1961), who have recently reported the commencement of a study of long-chain fatty acids (XXXVII) incorporating a selenium atom in various positions of the chain.

$$Me \cdot [CH_2]_n \cdot Se \cdot [CH_2]_m \cdot CO_2H, \text{ where } n + m = 12 \text{ or } 13.$$

$$\text{(XXXVII)}$$
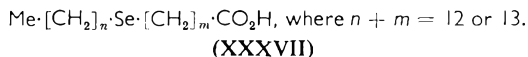
CONCLUSION

Whether organoselenium compounds will ever command a place in medicine is rather doubtful. However, the biological reports to date have shown that in certain spheres of activity, selenium compounds may be of some value. The indications are that this seems most likely in the antifungal, antibacterial and carcinostatic fields. It is encouraging to note that very recently at least two groups of workers have embarked on a comparative study of the metabolism of sulphur and selenium compounds. At present, the knowledge of the fate of selenium compounds in the body is limited and such studies can be expected to lead to better understanding of the biological role of selenium. Incorporation of selenium into animal proteins has already been demonstrated (McConnell and Wabnitz, 1957; McConnell, Roth and Dallam, 1959), while urinary excretion apparently follows the same route as for sulphur (McConnell, Kreamer and Roth, 1959).

One of the discouraging features of such studies, however, is the relative instability of selenium compounds. Thus, formulation can be expected to be extremely difficult. The fairly good lipid solubility, if a general characteristic, is likely to be an asset since the presence of water often accelerates decomposition, leading to breakdown products with powerfully unpleasant odours. Perhaps in this field more than in any other, the medicinal use of an active compound will be limited, not only by its toxic properties, but also by its physical, and hence sensory, stability.

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RESEARCH PAPERS

A COMPARISON OF THE THYROXINE : TRI-IODOTHYRONINE CONTENT AND BIOLOGICAL ACTIVITY OF THYROID FROM VARIOUS SPECIES

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Samples of ox and sheep thyroid were less potent than pig thyroid in biological assays when the doses were administered either on the basis of B.P. "Thyroxine-iodine" or U.S.P. total combined iodine. Chemical analyisis for iodothyronines revealed that the molar ratio of thyroxine to tri-iodothyronine approximated 2:1 in pig thyroid but approached 3:1 in ox and sheep preparations. Thus the ox and sheep samples which were less potent in the biological assay than pig thyroid also contained less tri-iodothyronine. By using tri-iodothyronine as the dosage basis, there was very little if any difference in physiological efficacy in goitre prevention assay.

IT has been established that the present official methods for the chemical assay of desiccated thyroid may not indicate the biological activity. Stasilli and Kroc (1956) have reported that on the basis of either the U.S.P. total combined iodine or the "Blau thyroxine-iodine", ox thyroid is much less active than pig thyroid in both the goitre-prevention and calorigenic assays in the adult rat. Similarly Johnson and Smith (1961) and Webb (1961) noted a lack of agreement between B.P. "thyroxine-iodine" values and physiological activity in preparations of pig, ox and sheep thyroid.

The present study was undertaken to determine whether the variation in biological potency reported for desiccated thyroid from different species could be explained on the basis of changes in content of thyroxine and tri-iodothyronine (liothyronine).

EXPERIMENTAL

Samples

An examination was made of representative beef and pork thyroid samples from an American source and sheep, ox and pig thyroid from a British supply. A highly purified pork thyroglobulin served as a primary reference standard. A sample of desiccated pork thyroid was used as a house standard and all assays were made against this preparation. Relevant details of the source and composition of the samples are given in Table I.

The biological activity of the thyroid products was determined orally by a goitre prevention assay (Wiberg and Stephenson, 1961) and subcutaneously by the mouse anoxia test (Burn, Finney and Goodwin, 1950).

The doses were administered on the basis of the "total combined iodine" (United States Pharmacopoeia XVI, p. 759) and were also computed in terms of "thyroxine-iodine" (British Pharmacopoeia, 1958, p. 678, Addendum, 1960, p. 60). The results of the assays were calculated by standard statistical procedures (Bliss, 1952; Emmens, 1948, Finney, 1952).

TABLE I

"THYROXINE IODINE" AND TOTAL IODINE CONTENT OF PREPARATIONS STUDIED

Preparation	B.P. "Thyroxine iodine" per cent	U.S.P. total combined iodine per cent	Ratio "Thyroxine iodine" / total iodine
Primary standard purified pig thyro-globulin[1]	0·24	0·86	0·279
House standard pig thyroid[2]	0·06	0·20	0·300
American samples—			
Pig[3]	0·26	0·79	0·329
Ox[4]	0·17	0·54	0·315
British samples—			
Pig[5]	0·20	0·57	0·351
Ox[5]	0·11	0·35	0·314
Sheep[5]	0·18	0·48	0·375

1. Warner Chilcott Laboratories. 2. Wilson Laboratories—this sample meets requirements of U.S.P XVI. 3. Armour Pharmaceutical Co.—a composited sample prepared from 7984 lb. of pig thyroids. 4. Armour Pharmaceutical Co.—a composited sample prepared from 3874 lb. of ox glands. 5. Burroughs Wellcome and Co.

In the chemical determination of the iodothyronines, the thyroid samples were hydrolysed by incubation with trypsin and erepsin, followed by chromatographic separation of a butanol extract of the hydrolysates (Devlin and Stephenson, 1962).

RESULTS

The combined results of three independent homogeneous assays are presented in Table II. In general, the total combined iodine and "thyroxine iodine" produced similar estimates of biological potency. The principal exception was the British sheep thyroid in which the assays based on the total iodine content were consistently higher than those employing the "thyroxine-iodine". This was observed with both the goitre prevention and mouse anoxia procedures. Reference to Table I, shows that the "thyroxine-iodine": total iodine ratio is highest in sheep thyroid which could account for these results.

A comparison of the relative potencies obtained from the two methods of biological assay also show good agreement. The British ox preparation indicated a higher level of activity in the goitre prevention test whereas the sheep thyroid was more potent when assayed by the mouse anoxia response. However, these particular results do not appear to be statistically significant.

Sheep and ox gland preparations, at equal concentrations of either total combined iodine or B.P. "thyroxine-iodine" were less potent physiologically than pig thyroid, with the one exception noted in Table II (sheep thyroid in the mouse anoxia assay based on total iodine content). This confirms the observations of Stasilli and Kroc (1956), Johnson and Smith (1961) and Webb (1961) in that the species of origin does affect the relative biological activity of desiccated thyroid.

TABLE II

A COMPARISON OF THE BIOLOGICAL ACTIVITIES OF DESICCATED THYROID PREPARATIONS
OBTAINED FROM DIFFERENT SPECIES

Preparation	Relative potency			
	Goitre prevention assay		Mouse anoxia assay	
	B.P. "Thyroxine iodine" per cent	U.S.P. total iodine $_3$ per cent	B.P. "Thyroxine iodine" per cent	US.P. total iodine per cent
Primary standard pork thyroglobulin	100	100	—	—
House standard desiccated pork thyroid	99·3 (94·0–107·4)	100·4 (95·3–105·9)	100	100
American samples—				
Pig	97·2 (93·7–100·6)	101·0 (96·0–108·0)	104·8 (80·1–137·2)	104·5 (79·9–136·7)
Ox	63·0 (56·3–70·3)	69·0 (61·6–77·2)	56·7 (45·2–71·3)	58·0 (46·2–72·5)
British samples—				
Pig	103·3 (95·9–111·4)	97·5 (91·0–104·3)	96·3 (80·5–115·3)	105·2 (88·2–126·3)
Ox	81·2 (74·7–88·7)	81·7 (75·2–88·8)	66·6 (49·2–90·2)	70·3 (52·1–95·5)
Sheep	73·8 (67·9–80·2)	86·6 (79·7–94·2)	88·1 (74·4–104·3)	104·1 (87·9–113·9)

Each value shown represents the weighted mean of three assays together with the range of potency in brackets.

The goitre prevention assay was superior to the mouse anoxia technique in precision, reproducibility and sensitivity. The Index of Precision (s/b) for the individual goitre prevention assay was always less than 0·2 and frequently below 0·1, whereas the mouse anoxia test produced Indices of Precision which lay between 0·35–0·45. The high residual error term found in the mouse anoxia assay was the major reason for the reduced precision. Transformation of the response parameter to either the reciprocal or to the log of the survival time not only reduced the error variance but also decreased the slope, and consequently there was no appreciable gain in the precision.

The thyroxine and liothyronine content of enzymic hydrolysates of the various thyroid samples is given in Table III. The liothyronine levels are lower in the ox and sheep samples than in preparations of pig thyroid. Thus it would seem that the molar ratio of thyroxine : liothyronine is about 2 : 1 in pork thyroid but approaches 3 : 1 in sheep and beef samples.

TABLE III

THYROXINE AND LIOTHYRONINE CONTENT OF DESICCATED THYROID SAMPLES

Preparation	Thyroxine		Liothyronine		Molar ratio[1] T_4/T_3
	mg./100 g.	micromoles/ 100 g.	mg./100 g.	micromoles/ 100 g.	
Primary standard pork thyroglobulin	230	296	108	166	1·78
House standard pork thyroid ..	47	60	19	29	2·06
American samples—					
Pig	212	273	73	112	2·43
Ox	116	149	35	53	2·80
British samples—					
Pig	146	188	75	115	1·64
Ox	119	154	38	58	2·65
Sheep	135	174	43	66	2·64

Each value shown is the mean of four determinations.
1. T_4 = Thyroxine. T_3 = Liothyronine.

The molar ratio (T_4/T_3) appears to be inversely related to the biological effectiveness of the dried thyroid samples. This observation suggested that liothyronine and not thyroxine might be responsible for the greater amount of the activity in the biological assays. Accordingly log dose response lines were plotted for the various goitre prevention assays on the basis of the liothyronine content of the samples. The results from one of these assays is presented in Fig. 1. For the sake of comparison,
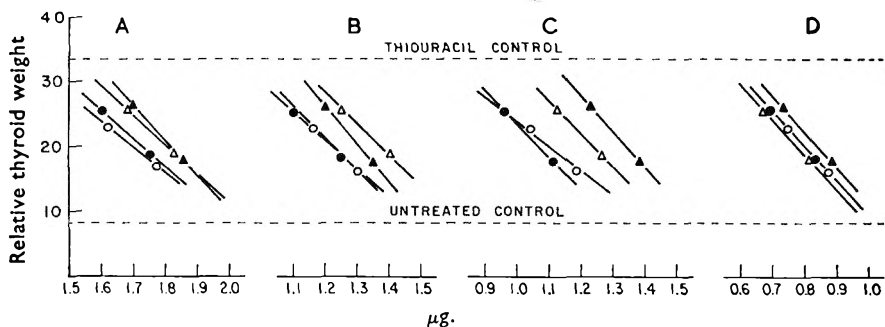
FIG. 1. Log. dose response lines showing the relation between the various parameters of thyroid dosage and response in the goitre prevention assay with adult female rats. Dosages are expressed as per 100 g. diet. Each point represents the mean of 10 animals. Samples assayed were: House standard ●, British pig ○, British ox △, and British sheep ▲.
A, U.S.P. total combined iodine. B, B.P. "thyroxine iodine." C, thyroxine. D, liothyronine.

the log dose response lines for the other iodine-measurements are also shown, namely the B.P. "thyroxine iodine", the U.S.P. total iodine and the thyroxine value as obtained by chromatographic resolution. Visual inspection of the graph indicates that only liothyronine removes this species difference in terms of the biological potency of the dried thyroid preparation. All the other assays produced similar results to those shown in Fig. 1. Hence it seemed of interest to recalculate these assays using the liothyronine content as the basis of dosage. These results are given in Table IV and it is evident that the various preparations now possess equivalent potency, in the goitre prevention test, regardless of species of origin. The obvious conclusion is therefore that liothyronine appears to provide the greater part of the biological activity when thyroid preparations are administered orally.

DISCUSSION

Since preparations of desiccated thyroid are prescribed exclusively as oral medication, it would seem advisable to use the same route for its biological assay. The route of administration in the mouse anoxia test is subcutaneous and this method of dosing may not evaluate certain variables which could affect the oral potency of a sample of thyroid powder. Some of these variables are, the thyroxine and liothyronine content, the rate of release of these two substances plus completeness of

digestion in the gastrointestinal tract and, the relative absorption of the liberated hormones from the gut.

Although the content of the active ingredients, thyroxine and liothyronine, can be obtained by chemical analysis, the second and third factors listed above could produce marked changes in the physiological availability of these compounds. Little is known about the rate of release of these substances and completeness of digestion but there is some evidence that the thyroid hormones are only partially absorbed through the intestinal wall. Frieden, Tukich and Winzler (1949) reported that the ratio of oral to parenteral potency for ($\pm$)-thyroxine was 1:2, using the goitre prevention response in rats. Other workers have found also

TABLE IV

RELATIVE BIOLOGICAL POTENCY OF DESICCATED THYROID SAMPLES FROM VARIOUS SPECIES BASED ON THE LIOTHYRONINE CONTENT

Preparation	T_3^1	Relative potency	Range of potency (P = 0.95)
House standard	19	100	—
American—			
Pig	73	96.1	92.6– 99.7
Ox	35	102.8	91.6–115.0
British—			
Pig	75	95.1	88.2–102.6
Ox	38	94.5	87.0–102.8
Sheep	43	105.3	96.9–114.2

Each value shown represents the weighted mean from three independent homogeneous assays employing the goitre prevention technique.
[1] Liothyronine concentration in mg./100 g. thyroid powder.

that (—)-thyroxine is less active by the oral route using a variety of assay techniques in different species. Kroc, Phillips, Stasilli and Malament (1954) noted oral dosing was only 34 per cent as effective in the antigoitrogenic assay and 26 per cent as effective in the calorigenic assay in rats compared to the subcutaneous route. Reineke, Travis and Kifer (1960) measured the suppression of the thyroid secretion rate in mink by (—)-thyroxine and concluded that only 34 per cent of the hormone was absorbed. In ruminants, the oral potency of thyroxine is even lower. Mixner and Lennon (1960) found 10–15 per cent of thyroxine is absorbed by lactating dairy cattle based on the protein bound iodine values while Turner and Reineke (1946) estimated that around 5 per cent of the oral dose is physiologically available in sheep based on a weight loss assay.

Desiccated thyroid samples do not show a comparable loss of oral potency in the goitre prevention assays as that recorded for (—)-thyroxine. Friedin and others (1949) and Kroc and others (1954) indicate desiccated thyroid is from 70–75 per cent as active by the oral route as that obtained by subcutaneous injection. A plausible explanation for this higher ratio of oral to parenteral potency for thyroid powder can be adduced from the results published by Gross and Pitt-Rivers (1953). These workers found that oral thyroxine had 39 per cent of the potency of the same parenteral dose whereas orally-administered liothyronine retained 86 per cent of the parenteral activity. Thus the greater extent of absorption of liothyronine coupled with its higher biological activity

could mean that the liothyronine content of desiccated thyroid is quantitatively much more important than the thyroxine level. This possibility has been discussed in detail by Levy and Knox (1961).

The feasibility of the hypothesis that liothyronine contributes the major portion of the biological activity of desiccated thyroid appears to be warranted on the basis of the results presented in Table IV. This does not exclude thyroxine as an active ingredient of dried thyroid but its role may be of minor importance. Since no firm values are available for the amounts of thyroxine and liothyronine released during digestion and then absorbed, it would be highly speculative to assign any definite fraction of the biological activity to the thyroxine content of the thyroid sample. In addition the species of origin would affect this value, since the relative liothyronine levels were lower in ox and sheep preparations. However, in consideration of the fact that liothyronine is from 3 to 8 times as potent as thyroxine (Gross and Pitt-Rivers, 1954; Tomich and Woollett, 1953; Stasilli, Kroc and Meltzer, 1959) the latter may contribute as little as 5 per cent and as much as 30 per cent of the total biological response.

These studies have been made with laboratory or domestic animals and it is quite possible that the digestion of desiccated thyroid by man and the subsequent absorption of the hormones follow a different pattern. Therefore the potency values reported in Table IV based on the liothyronine content of the thyroid samples may not be indicative of the therapeutic effectiveness in humans.

However, it is evident that the present chemical assays of the United States and British Pharmacopoeias do not always reflect the biological activity of desiccated thyroid. The goitre prevention assay involving the oral administration of the test preparations to rats seems to offer a more fruitful procedure at the present time for assessing physiological activity.

If the contributions of each of the active thyroid constituents to the overall biological response could be established with some degree of reliability, then it is quite possible that a chemical assay which measures thyroxine and liothyronine might obviate the present need for biological assay.

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THE NATURE OF RESISTANCE OF A PENICILLIN TO HYDROLYSIS BY PENICILLINASE

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5-Methyl-3-phenyl-4-isoxazolyl penicillin is a competitive inhibitor of penicillinase. Interaction with this compound causes marked conformational changes in the enzyme. The changes are reflected in increased susceptibility to iodination, urea and heat. Present observations are related to previously recorded evidence, and the conclusion is reached that the side-chain of a penicillin may confer resistance by distorting the catalytic orientation of penicillinase.

PENICILLINASE hydrolyses the β-lactam ring in 6-aminopenicillanic acid and in many of its derivatives, including clinically important penicillins such as benzylpenicillin and the phenoxyalkyl penicillins. The widespread occurrence of penicillinase producing bacteria has prompted attempts to synthesise new derivatives of 6-aminopenicillanic acid which would combine high antibiotic potency with resistance to hydrolysis by penicillinase. One such derivative, 6-(2,6-dimethoxybenzamido)penicillanic acid (methicillin) (Rolinson, Batchelor, Stevens, Cameron-Wood, and Chain, 1960) has already proved very effective in clinical practice.

Experiments with methicillin have yielded information which may explain the structural basis of resistance to penicillinase. There is now good evidence that the active site of penicillinase acquires a specific catalytic orientation when in contact with a hydrolysable penicillin (Citri and Garber, 1958; Citri and Garber, 1960; Citri, 1960). In contrast, interaction with methicillin causes loss of the specific conformation which is apparently essential for enzymic activity (Citri and Garber, 1961; Garber and Citri, 1962).

Our experiments with 5-methyl-3-phenyl-4-isoxazolyl penicillin, [P-12] here presented, indicate that such distorting effect may indeed account for the resistance of some penicillins to hydrolysis by penicillinase.

EXPERIMENTAL

Penicillinase. The penicillinase was prepared from the culture supernatant of strain 569/H of *Bacillus cereus*, grown as previously described (Citri and Garber, 1960) and the supernatant enzyme concentrated and purified by the procedure described elsewhere (Citri, Garber and Sela, 1960). The specific enzymic activity of the penicillinase preparations used throughout this work was similar to that reported by Kogut, Pollock and Tridgell (1956) for crystalline penicillinase of *B. cereus*.

Assay of penicillinase. Total penicillinase activity was assayed by the manometric method of Henry and Housewright (1947). The iodine

resistant (α-type) activity was assayed directly by the iodometric procedure previously described (Citri, Garber and Sela, 1960).

Iodination of penicillinase. The iodinating reagent consisted of 0·003 M I$_2$ and 0·02 M KI (final concentration). The treatment exposed the enzyme to the iodinating reagent for 5 min. at 0° and pH 7·3.

Heat treatment. The heat treatment was by immersing test tubes in a thermostatic water-bath. The temperature was regulated by Thermonix II Immersion Thermostat within ±0·1°. At the end of the incubation the tubes were immersed in an ice-bath for 1 min. and transferred to a 30° water-bath where the enzyme was assayed.
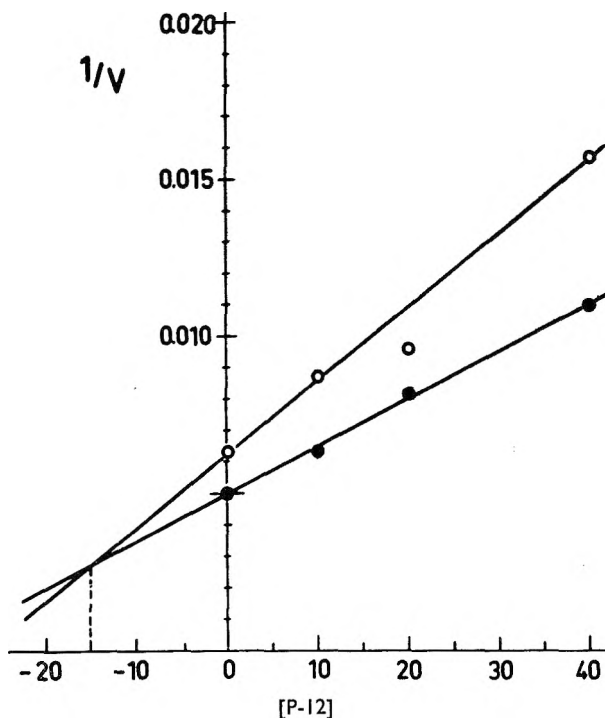
Fig. 1. Competitive inhibition of penicillinase by P-12. Initial velocities for the hydrolysis of benzylpenicillin in the presence of P-12, plotted according to the method of Dixon (1953). The assay system contained 0·05 μg. of penicillinase protein, 3–10 μM benzylpenicillin and 10–40 μM P-12 in a total volume of 3·0 ml. The reactions were run in a Warburg respirometer under conditions used in the manometric assay of penicillinase.

○—○   3 μM benzylpenicillin.   ●—●   10 μM benzylpenicillin.

Assay of residual activity. The assay was based on the iodometric procedure for the determination of iodine resistant (α-type) activity (Citri and Garber, 1958). The reagent mixture (0·5 ml. of I$_2$, 0·025M in 0·125M KI, 1·0 ml of 0·1M phosphate buffer, 3·0 ml. of 0·5 per cent gelatin and 3 mg. of benzylpenicillin) was kept for 5 min. at 30° before the assay. The assay was started by the transfer of the reagent mixture into the

tube containing the treated preparation. The total volume of the assay was 5 ml.

In the assay of iodinated samples of penicillinase the iodine content of the assay reagents was reduced by the amount used for iodination. Hence the iodine concentration used in the assay was identical in all samples and equal to that used in the original procedure.

Penicillins. Sodium benzylpenicillin B.P. was obtained from Merck and Co., Inc.

Sodium 5-methyl-3-phenyl-4-isoxazolyl penicillin monohydrate [P-12] was supplied by Bristol Laboratories.

FIG. 2. Sensitisation of penicillinase to iodine as a function of P-12 concentration. Penicillinase (0·5 µg. of enzyme protein) was exposed to varying concentrations of P-12 in the presence of iodine (0·0038M I₂ in 0·02M KI) (Curve A) and in its absence (Curve B). The treatment was at 0° C. in 1·0 ml. of 0·006M phosphate buffer at pH 7·3. At the end of 5 min. the reactants were diluted with an excess of the substrate (3,000 µg. benzylpenicillin in 4 ml. of 0·03M phosphate buffer pH 7·0 containing 0·4 per cent gelatin). The rate of hydrolysis of benzylpenicillin by the residual enzyme was immediately assayed.

RESULTS

Inhibition of penicillinase by P-12. P-12 is highly resistant to hydrolysis by penicillinase of *B. cereus.* The low rate of hydrolysis of P-12 compared with benzylpenicillin has been previously reported (Gourevitch and others, 1961). As with methicillin (Garber and Citri, 1962), P-12 was found to inhibit competitively the hydrolysis of benzylpenicillin by *B. cereus* penicillinase. The kinetics of the inhibition have been investigated with two concentrations of benzylpenicillin and a series of concentration of

786

P-12. The results, plotted according to the method of Dixon (1953) are presented in Fig. 1. The K_i value obtained from the Dixon plot is 5×10^{-3}M. This value is quite close to that obtained for methicillin ($K_i = 1.8 \times 10^{-3}$M) (Garber and Citri, 1962).
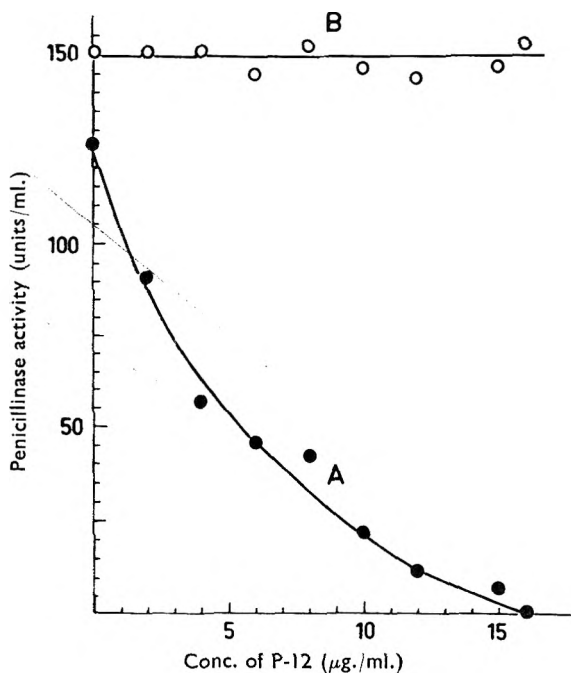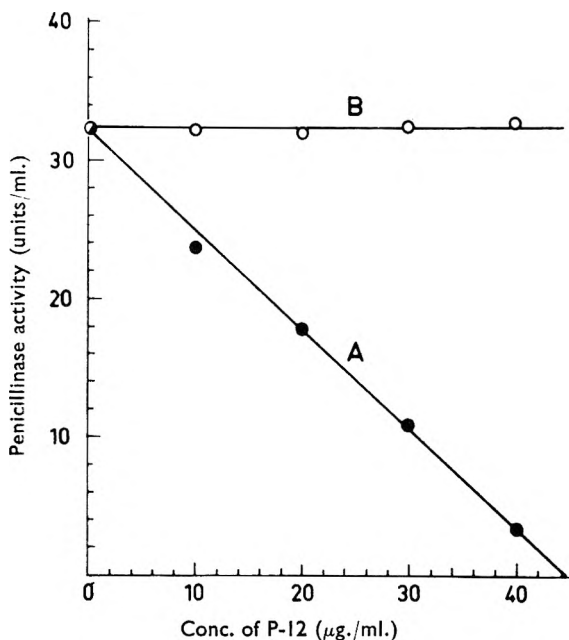

FIG. 3. Sensitisation of penicillinase to urea as a function of P-12 concentration. Penicillinase (0.14 μg. of enzyme protein) was exposed for 5 min. at 0° C to increasing concentrations of P-12. The treatment was in 3.0 ml. phosphate buffer (0.25M, pH 7.0) containing 0.5 per cent gelatin, in the presence of 4M urea (A) and without urea (B). Assay was started by adding benzylpenicillin (3,000 μg. in 1.0 ml. of 0.1M phosphate buffer pH 7.0) and iodine (0.5 ml. of I_2, 0.0025M in 0.125M KI) to the treated samples.

Sensitisation of penicillinase to iodination. Changes in the susceptibility to iodination have been found to provide a sensitive indicator of structural changes in the molecule of penicillinase (Citri, 1958; Citri, Garber and Sela, 1960). Using this criterion the effect of methicillin was demonstrated on the conformation of penicillinase (Citri and Garber, 1961; Garber and Citri, 1962).

P-12 affects the sensitivity of penicillinase in the same way as methicillin as shown in the following experiment.

The enzyme was exposed to varying concentrations of P-12 in the presence of iodine and in its absence. The residual activity of the treated enzyme was assayed, and the results are summarised in Fig. 2. It will be noted that whereas exposure of penicillinase to P-12 alone had no effect (Fig. 2,B), exposure to P-12 in the presence of iodine caused a loss in activity which was proportional to the concentration of P-12 (Fig. 2,A).

Potentiation of the effect of urea on penicillinase. The effect of P-12 on the reactivity of penicillinase is similar to that observed with moderate concentrations of hydrogen bond breaking agents such as urea or guanidine hydrochloride (Citri and Garber, 1958; Citri, Garber and Sela, 1960). The susceptibility to iodine induced by urea is reversed by the substrate, but the reversal is precluded if iodine is present before or during the assay. Hence pre-treatment with adequate concentrations of urea causes a loss of enzymic activity under the present conditions of assay. No such loss is noticeable after a short exposure to 4M urea in the cold. If, however, P-12 is present during this treatment the enzyme becomes inactivated. The loss of activity is directly proportional to the concentration of P-12, as will be apparent from the results presented in Fig. 3.
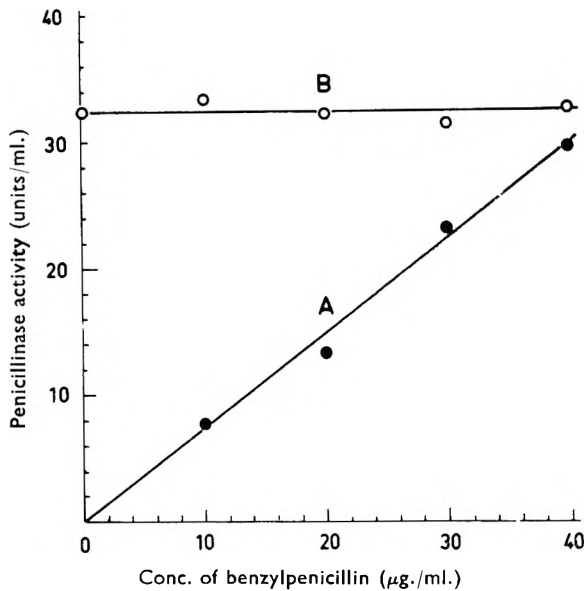
FIG. 4. Reversal of the effect of P-12 by benzylpenicillin. Penicillinase (0·14 µg. of enzyme protein) was exposed for 5 min. at 0° C. to P-12 (50 µg./ml.) and increasing concentrations of benzylpenicillin. Treatment was carried out in the presence of 4M urea (A) and without urea (B). For other experimental conditions see Legend to Fig. 3.

Reversal of the effect of P-12 by benzylpenicillin. As shown previously (Fig. 1) P-12 inhibits competitively the enzymic hydrolysis of benzyl-penicillin. It follows that P-12 and benzylpenicillin combine with the same site of the enzyme. It would be expected, therefore, that the interaction of P-12 with penicillinase which lowers resistance to urea would be affected by benzylpenicillin. Since benzylpenicillin is known to reverse the effect of urea, presumably by favouring the active con-formation of penicillinase (Citri and Garber, 1960), it was actually expected to reverse competitively the effect of P-12 under the present conditions. Results demonstrating reversal by the substrate are shown

in Fig. 4, where the activity of penicillinase pre-treated with urea and P-12 is plotted against the concentration of benzylpenicillin present during the pre-treatment. It will be noted that the survival of penicillinase activity is proportional to the ratio of substrate to analogue during the pre-treatment with urea.
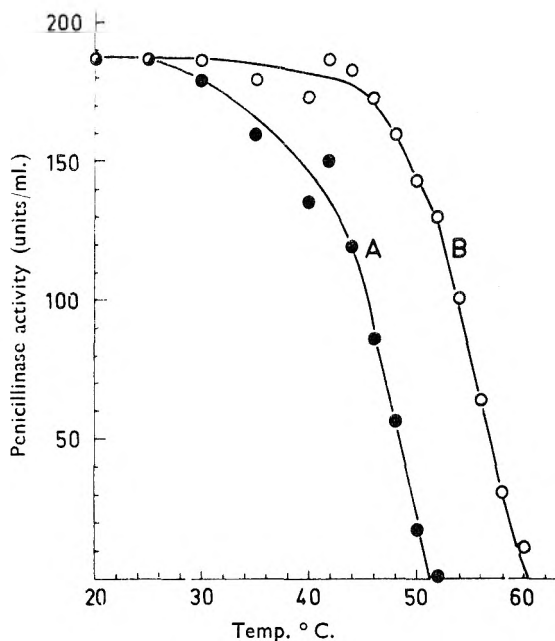
FIG. 5. Effect of P-12 on the thermostability of penicillinase. Reaction mixture (final volume — 0·4 ml.) consisted of penicillinase — 0·5 μg.; phosphate buffer pH 7·3 — 0·05M; gelatin — 0·25 per cent and P-12 — 0·18 μg. (Curve A). Controls contained no analogue (Curve B). At the end of 2 min. exposure to the indicated temperatures the residual activity was determined as described in the Experimental Section.

Potentiation of the effect of heat on penicillinase. The results presented so far indicate that the interaction between penicillinase and P-12 involves breaking of some specific secondary links, such as hydrogen bonds, in the vicinity of the active site. According to this interpretation, P-12 would be expected to lower the thermostability of penicillinase.

The effect of substrate analogues on the stability of the enzyme to heat has been the subject of more experiments (Citri and Garber, 1962). Substrate analogues do indeed cause increased sensitivity to heat, as shown in Fig. 5, where the effect of P-12 is presented as a function of temperature of incubation. The P-12 induced thermolability is proportional to the concentration of the analogue. This is shown in Fig. 6 where the survival of enzyme activity at 45 and 48° is plotted against the concentration of P-12 present during the treatment.

Effect of pH on the thermostability of penicillinase in the presence of P-12. The pattern of thermostability of penicillinase over the range of pH 4–7, shown in Fig. 7 (Curve B) is completely changed in the presence of P-12 (Fig. 7, A). There is practically no loss of activity after 2 min. exposure to 50° at acid pH (pH 4·0–6·0) unless P-12 is present. With P-12 added complete inactivation takes place at pH 4·0–4·5. The thermostability of penicillinase in the presence of P-12 increases sharply in the range of pH 4·5–5·0, and decreases soon after. Again P-12 causes complete inactivation at pH 7·0 when the control enzyme, although not completely stable, retains most of its activity. The curve obtained with P-12 at 50° is similar to that obtained with the enzyme alone at 58°, and in particular to one obtained with methicillin at 48° (Citri and Garber, 1962).
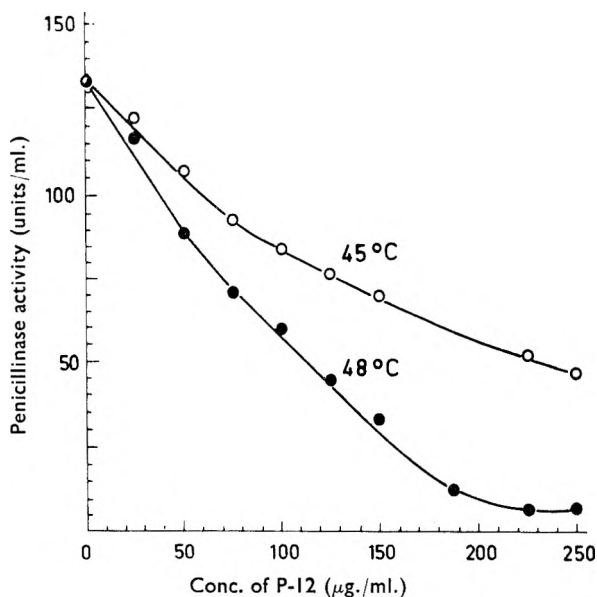
FIG. 6. Thermal inactivation of penicillinase as a function of P-12 concentration. Reaction mixtures (final volume—0·4 ml.) consisted of: penicillinase—0·5 μg.; phosphate buffer pH 7·3—0·05M; gelatin—0·25 per cent, and varying amounts of P-12. At the end of 2 min. exposure to 45° C. (○) and 48° C. (●) respectively, the residual activity was measured as described in the Experimental Section.

DISCUSSION

The penicillin chosen for this study represents a new class of derivatives of 6-aminopenicillanic acid, the 4-isoxazolylpenicillins. When suitably substituted in both *ortho* positions, members of this class are highly resistant to hydrolysis by penicillinase (Doyle, Long, Nayler and Stove, 1961). The other class of penicillins known to be resistant to penicillinase, has been represented by methicillin in a series of similar studies (Citri and Garber, 1961; Garber and Citri, 1962).

A comparison of properties common to both types of penicillins with those of hydrolysable penicillins will provide a basis for discussing the following questions.

What are the distinctive structural features of a resistant penicillin?

By what mechanism do such distinctive features protect the β-lactam ring from enzymic hydrolysis?

The first question can be answered by comparing the structural formulae representing (I), a class of hydrolysable penicillins (α-phenoxyalkyl penicillins), and both classes of resistant penicillins: a 4-isoxazolyl penicillin (II) and an aromatic disubstituted analogue (III).

I

II

(In P-12, R = Ph, R' = Me)

III
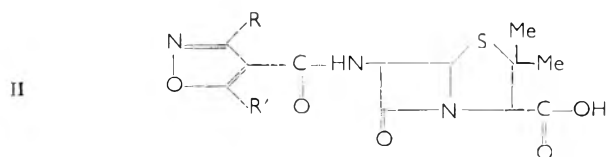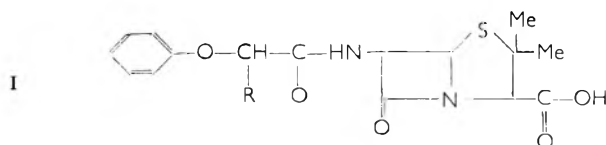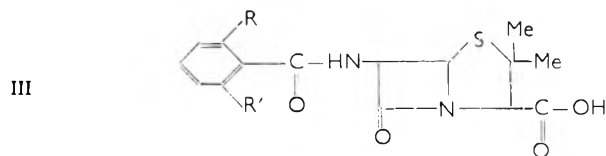
(In methicillin R = R' = OMe)

The various penicillins differ, by definition, in the side-chain alone. Hence it is a modification in this moiety that imparts resistance to enzymic hydrolysis. It will be obvious from comparing the respective structures, that the side-chain of a resistant penicillin contains a ring situated in close proximity to the nucleus and substituted in both *ortho* positions. Some very interesting observations on the effect of various substituents in both groups of resistant penicillins, have recently been reported (Doyle, Long, Nayler and Stove, 1961).

In this paper we are concerned specifically with the second question, namely that of the mechanism by which the side-chain of a penicillin affects the action of penicillinase. Probably the most acceptable assumption is that the side-chain determines whether a penicillin can be properly accommodated on the active site of the enzyme. Although stated in rather general terms this is usually taken to imply that the active site is a rigid structure made to fit the substrate. Accordingly bulky substituents in the side-chain will prevent access of a substrate analogue to the active site.

Attempts to explain the properties of methicillin have been based on such a model (e.g. Knox, 1961), although there is no evidence to support it. On the other hand, there is a good evidence for an alternative concept. According to this concept, which may be regarded as a special case of the "induced fit" theory (Koshland, 1959), the alignment necessary for the activity of penicillinase depends on the flexibility of the active site, and on the orienting effect of the side-chain.
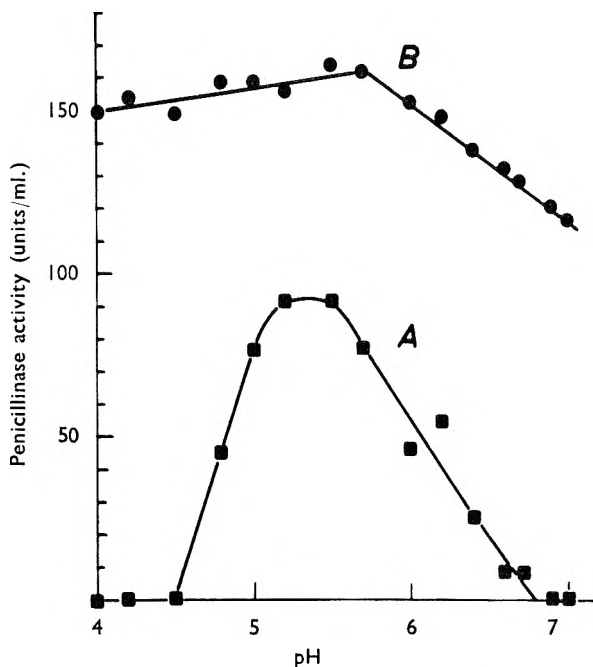
FIG. 7. Survival of penicillinase activity following heat treatment at varying pH. Aliquots of penicillinase (0·55 μg. of enzyme protein) in 0·05M buffer solutions of varying pH, exposed for 2 min. to 50° C. (A) in the presence of P-12 and (B) in its absence. Total volume—0·4 ml. Buffers used: phthalate—NaOH (pH 4·0–6·0), phosphate (pH 6·0–7·5). For assay of residual activity see Experimental Section.

The evidence has been presented and discussed in our previous reports (see refs.). We wish to emphasise that the effects of the isoxazolyl penicillin, used in this study, on the conformation of penicillinase are virtually identical with those observed previously with methicillin. In either case the change in conformation is reversed by hydrolysable penicillins.

The distinctive effect of compounds representing both classes of resistant penicillins on the conformation of the active site of penicillinase points to a common mechanism responsible for the resistance. The following is a tentative formulation of our conclusion concerning the nature of resistance to penicillinase. The enzymic activity of penicillinase depends on a definite and precise orientation of the active site. Such

orientation is imparted by some penicillins and distorted by others, according to the structure of the side-chain. Consequently, the former penicillins are classed as substrates, whereas the latter are relatively immune to enzymic hydrolysis.

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A METHOD FOR THE ESTIMATION OF ADRENALINE AND NORADRENALINE IN URINE

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A method for the estimation of adrenaline and noradrenaline in urine is described. The catecholamines are adsorbed cn aluminium oxide and eluted with acid. The adrenaline and noradrenaline are estimated separately after oxidation with manganese dioxide at different pH values and conversion to the trihydroxyindole derivative for fluorimetric estimation. A mean recovery of 82 per cent (± 5 per cent S.D.) was obtained for total amines estimated as noradrenaline, adrenaline recovery was 83 per cent (± 6 per cent S.D.) and noradrenaline recovery was 80 per cent (± 6 per cent S.D.). The results were in good agreement with biological assay results.

MODERN methods of estimation of adrenaline and noradrenaline in urine involve adsorption of the amines on aluminium oxide (Pitkanen, 1956; Lund, 1952) or on an ion-exchange resin (Weil-Malherbe and Bone, 1957; Crawford and Law, 1958; Wright, 1958). The amines are then eluted and estimated biologically (Euler and Hellner, 1951; Burn, 1953) or fluorimetrically (Lund, 1952; Crawford and Law, 1958; Weil-Malherbe and Bone, 1952; Euler and Floding, 1955).

Attempts to use existing fluorimetric methods of assay have not been entirely satisfactory in our laboratories, results frequently differing from concurrent biological assays, inconsistent recoveries and anomalous high results occasionally being obtained.

This paper describes a modification of Lund's (1952) fluorimetric method for the estimation of adrenaline and noradrenaline.

EXPERIMENTAL AND RESULTS

Reagents

Aluminium oxide (Chromatographic B.D.H.); adrenaline solution, 1 in 1,000 (Burroughs Wellcome); Ascorbic acid, B.P., which was used to prepare a 1 per cent aqueous solution and was not kept longer than 5 days at $0°$; Manganese dioxide (B.D.H.), technical grade, pretreated as described by Crawford and Law (1958); noradrenaline solution, 1 in 1,000 (Levophed, Bayer); buffer solution, pH 3·5–50 ml. 0·2M potassium hydrogen phthalate plus 7·9 ml. 0·2N hydrochloric acid: buffer solution, pH 6·5–0·1M disodium hydrogen phosphate; freshly distilled water. All other reagents were of Analar quality.

Extraction

Urine (100 ml.), ethylenediaminetetra-acetate (10 to 20 mg.) and aluminium oxide (4 g.) were stirred magnetically. Sodium hydroxide was added over 2 min. to give a blue colour to thymol blue paper (about

pH 8·5). Stirring was continued for a further 6 min. and the mixture was then centrifuged at 2,000 r.p.m. for 2 min.

The precipitate was washed with two quantities of 50 ml. of distilled water. Elution was with two portions, each of 10 ml. of N sulphuric acid and separated by centrifugation. Elution was repeated with a further three quantities each of 10 ml. of distilled water. All eluates were then pooled.

Estimation of Total Amines as Noradrenaline

Five ml. of the pooled eluates was mixed with 25 ml. of 0·1M disodium hydrogen phosphate solution. The precipitate formed adsorbed catecholamines, but these are still susceptible to oxidation and the resultant adrenochrome is not adsorbed by the precipitate.

This solution, at pH 6·5, was divided into three quantities of 10 ml. in three 50 ml. centrifuge tubes, designated standard, test and blank. Hydrochloric acid (1 ml., 0·01N) was added to the test and blank tubes and the same quantity containing 0·1 μg. of noradrenaline was added to to the standard tube. Manganese dioxide (100 mg.) was added to each tube which was then shaken continuously by hand for exactly 45 sec. and centrifuged for 2 min.

Nine ml. of clear supernatant from each tube was transferred to 15 ml. centrifuge tubes designated standard, test and blank. To the test and standard tubes, ascorbic acid solution (0·15 ml., 1 per cent) was added. Sodium hydroxide solution (0·85 ml., 5N) was added to all three tubes which were closed by rubber stoppers and inverted three times to mix. After exactly 4 min. ascorbic acid solution (0·15 ml., 1 per cent) was added to the blank and, after mixing by inversion, all three tubes were centrifuged for 6 min. to remove the traces of brown mucilaginous precipitate which appeared. The supernatant was pipetted off. The fluorescence of test, blank and standard solutions was compared in a direct reading E.I.L. fluorimeter using a Chance OB 10 primary filter and a Chance OY 2 secondary filter.

Estimation of Adrenaline

To a 5 ml. portion of the pooled eluate was added buffer solution (25 ml., pH 3·5). The solution was then divided into three 9 ml. portions designated standard, test and blank. Adrenaline (0·1 μg. in 1·0 ml. of 0·01N hydrochloric acid) was added to the standard and hydrochloric acid (1·0 ml., 0·01N) was added to the test and blank solutions. The solutions were then inverted to mix and the standard and test solutions were oxidised with 100 mg. of manganese dioxide for 45 sec. After centrifuging all three tubes for 2 min., the fluorescent derivative was prepared by the addition of ascorbic acid solution (0·15 ml., 1 per cent) followed by 0·85 ml. of 5N sodium hydroxide solution. The fluorescence was then examined as before.

After determination of the relative fluorescence of adrenaline to noradrenaline at pH 6·5, the noradrenaline content was calculated.

Demonstration of linear relationship of concentration of adrenaline and noradrenaline to fluorescence. A series of concentrations of adrenaline and noradrenaline in separate solutions, pH 6·5, were estimated fluorimetrically. A linear relationship between concentration and fluorescence was demonstrated for adrenaline and noradrenaline at concentrations between 0·025 and 0·20 μg./ml.

Estimation of adrenaline and noradrenaline in pure solution. In a series of ten estimations, the mean recovery for total amines as noradrenaline was 81·7 per cent (S.D. $\pm$ 0·08 per cent), noradrenaline 80·3 per cent (S.D. $\pm$ 0·18 per cent) and adrenaline 83·1 per cent (S.D. $\pm$ 0·10 per cent).

Recovery of adrenaline and noradrenaline added to urine. Adrenaline (5 μg.) and noradrenaline (50 μg.) were added to 1,000 ml. of urine of known adrenaline and noradrenaline content. After estimating the adrenaline and noradrenaline content the percentage recovery was determined.

In a series of ten estimations the mean recovery for total amines as noradrenaline was 81·7 per cent (S.D. $\pm$ 4·8 per cent), noradrenaline 80·3 per cent (S.D. $\pm$ 6·3 per cent) and adrenaline 83·1 per cent (S.D. $\pm$ 6·3 per cent).

Comparison of results obtained with results of bioassay on the same sample of urine. Concurrent fluorimetric and biological estimations were carried out on urine samples from 25 hypertensive patients. Total amines were determined biologically as noradrenaline using a cat blood-pressure method (Willoughby, 1958). The mean fluorimetric results were 99·3 per cent of the mean biological results (S.D. $\pm$ 13·4 per cent).

Determination of the specificity of the method. A solution was prepared containing adrenaline (1 μg.), noradrenaline (5 μg.), DOPA (1 μg.) and dopamine (50 μg.) in 50 ml. of 1·0N hydrochloric acid. A 10 ml. sample was extracted and estimated and found to contain 0·8 μg. adrenaline and 4·3 μg. noradrenaline in 50 ml. of the original solution. This suggests that DOPA and dopamine do not interfere in this fluorimetric estimation.

DISCUSSION

Lund's original method has been modified in an attempt to develop a method which gave the same results as those obtained by biological assay.

Recovery has been improved by the addition of EDTA, slow addition of alkali and rapid magnetic stirring. Under these conditions, breakdown of adrenaline and noradrenaline is minimal and constant.

Substances which caused quenching or potentiation of fluorescence were a source of error in initial experiments. The use of internal standards of adrenaline at pH 3·0 and noradrenaline at pH 6·5 has removed this source of error.

Non-specific fluorescence, extracted from the urine with the catecholamines, interfered with the preparation of the blanks as it faded in a similar manner to adrenolutin and noradrenolutin. The blank at pH 3·0

was therefore prepared by omitting the oxidation. At pH 6·5, fading of the blank was prevented, after a 4 min. interval, by the addition of ascorbic acid solution.

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THE ESTIMATION OF *N*-BENZYL-*N'N''*-DIMETHYLGUANIDINE (BW 467C60) IN URINE AND SOME OBSERVATIONS ON ITS REACTION WITH HYPOBROMITE

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N-Benzyl-*N'N''*-dimethylguanidine, a new anti-adrenergic drug, was estimated in urine by treatment with alkaline hypobromite, extraction of the bromo derivative with carbon tetrachloride and spectrophotometric estimation of the iodine liberated from acidified potassium iodide. Some peculiarities of the bromo derivative are described.

N-BENZYL-*N'N''*-DIMETHYLGUANIDINE (BW 467C60; I) is a new anti-adrenergic drug with properties resembling both bretylium and guanethidine (Boura and Green, 1962). Being a trisubstituted guanidine, the methods used for estimation of monosubstituted guanidines were not

$$\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{N}=\text{C}\underset{\text{NHMe}}{\overset{\text{NHMe}}{\diagdown}}$$

(I)
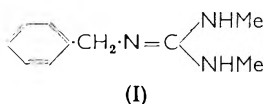
applicable to its assay, but during attempts to modify the Sakaguchi test it was found that addition of alkaline hypobromite to an aqueous solution of the drug gave a product that was readily extracted by chloroform or carbon tetrachloride. This product behaved like an *N*-bromo compound and liberated iodine from acidified potassium iodide. The iodine liberated was a measure of the amount of drug in the original solution but only one instead of the two atoms of iodine expected was liberated per molecule of drug. This result, and the possibility that the bromo derivative might serve to isolate and purify the drug excreted in urine, led to further examination of its chemistry. This proved to be unexpectedly complicated.

The *N*-bromo compound decomposed in carbon tetrachloride solution at room temperature to give appreciable amounts of benzyl bromide, methylamine and carbon dioxide. This decomposition, reminiscent of the Hofmann degradation of acid amides, did not occur at 0° and the product could be isolated as a yellow solid which was stable when stored at this temperature. It was decomposed by acidified potassium bromide, but not apparently by hydrobromic acid, in a complicated manner. In this reaction rather more than two moles of hydrogen bromide were absorbed per mole of bromo compound with elimination of a small proportion of methylamine and the evolution of about one-third mole of carbon dioxide. Little bromine was produced. The main reaction product had properties suggesting a quaternary ammonium salt of the

empirical formula $C_{14}H_{23}Br_2N_4O_2$.   It lacked any readily observable effect in rats at 30 mg./kg. intraperitoneally and since the determination of its structure was likely to be prolonged the investigation was discontinued.

EXPERIMENTAL

BW 467C60 iodide and sulphate were supplied by Dr. E. Walton.

Chromatograms were developed in s-butanol:acetic acid:water (11:5:3).   The drug could be visualised on paper by Dragendorff's reagent (sensitivity about 30 μg.).

Assay of BW 467C60 in urine.   Bromine (0·5 ml.) was dissolved in freshly prepared 2N-sodium hydroxide (50 ml.) as required.   Potassium iodide (0·5 g.) was dissolved in 0·02N-sodium hydroxide (25 ml.).

The sample of urine (15 to 30 ml.) was adjusted to pH 7 and passed down a column (10 × 1 cm.) of Zeo-Karb 226 (H⁺) and the column washed with water to remove interfering substances, mainly urea and creatinine. Bases were eluted with 0·1N hydrochloric acid (17 ml.), the eluate neutralised by 2N-sodium hydroxide (freshly made) and the volume adjusted to 20 ml.   Aliquots (6 ml.) were taken for assay.   To each was added carbon tetrachloride (5 ml.) followed by alkaline hypobromite (0·2 ml.). The mixture was shaken for a few min., lightly centrifuged and 4 ml. of the solvent layer pipetted into a tube containing potassium iodide solution (0·1 ml.).   2N-Sulphuric acid (0·3 ml.) was added, the mixture shaken, lightly centrifuged, and iodine in the carbon tetrachloride estimated at 510 mμ.

Carbon dioxide inhibited the liberation of iodine.   The bromo derivative could be extracted by chloroform but it was unstable in this solvent. Warming with chloroform and alkali led to the odour of carbylamines.

Spectrophotometric assay of iodine in carbon tetrachloride was possible down to 10 μg./ml. (optical density about 0·03 for a 1 cm. light path). This is equivalent to about 300 μg. of BW 467C60 iodide in the urine sample treated as described.

By comparison of the amount of iodine liberated by a known amount of BW 467C60 with a standard solution of iodine in carbon tetrachloride it was found that 1 mole drug corresponded to 1 atom iodine (Found: 1·04) instead of the 2 atoms expected.   This result was confirmed by iodometric titration (Found: 0·92 moles sodium thiosulphate per mole BW 467C60 sulphate).

Recovery of 1 mg. quantities of drug averaged 80 ± 4 per cent in 4 trials using aqueous solutions.   Recovery of 8 mg. added to normal urine was 85, 90 per cent in 2 trials.   Normal urine gave negligible blank values.

Excretion of BW 467C60 by patients.   Urine was collected from a subject who had received 40 mg. of the sulphate orally.   Table I shows the progress of excretion during 12 hr.

Table II shows the daily excretion of the drug by a patient who received 45 mg. of the sulphate daily (40 mg. on the first day).   To check the assay, 50 μg. of drug was added to 25 ml. of the day 6 sample.   Recovery of the additional drug was 52 μg.

Experiments with Brominated BW 467C60

At room temperature. BW 467C60 sulphate (1 g.) with a trace of α-benzyl-^{14}C-labelled drug in water (5 ml.) was shaken with hypobromite (70 ml. ≡ 0·7 ml. bromine) and carbon tetrachloride (10 ml.). The product was extracted with further portions of solvent. The extract was yellow and soon deposited a white solid while acquiring a pungent odour and lachrymatory property due to benzyl bromide. The aqueous layer, which contained 34 per cent of the added ^{14}C, smelled of amines, and when distilled gave bases equivalent to 518 ml. N acid per mole drug. On cooling, the solution in the distilling flask deposited white plates m.p., 82–84°, which were strongly radioactive. The distilled base was identified as methylamine by conversion to the picrate, m.p. 206–211° (no depression with authentic material). Solids in the carbon tetrachloride were redissolved by a little ethanol. 2N-Sulphuric acid (10 ml.) was added, followed by gradual addition of potassium bromide (4 g. in water, 40 ml.). Gas containing carbon dioxide (baryta) was evolved at each addition of bromide and the mixture assumed a deep orange colour but there was no precipitate. The aqueous phase contained 60 per cent of the added ^{14}C, leaving a trace in the solvent. The aqueous layer when basified yielded 0·14 g. of a basic oil to ether. Using manometric methods it was shown that 1 mole drug eliminated 1 mole carbon dioxide.

TABLE I

EXCRETION OF BW 467C60 IN URINE AFTER A SINGLE ORAL DOSE (40 MG.) OF THE SULPHATE

Collection interval	80 min.	167 min.	173 min.	85 min.	215 min.	Aggregate (12 hr.)
Urine vol. (ml.) ..	192	97	165	210	580	—
Mg. drug ..	0·32	0·66	1·52	1·29	3·54	7·33
μg./min. ..	4·0	4·0	8·8	15·2	16·5	—

TABLE II

EXCRETION OF BW 467C60 IN URINE DURING PROLONGED ORAL DOSAGE 40 mg. of the sulphate was given in the first day, and 45 mg. daily subsequently

Days	1	2	3	4	5	6	7
mg. ..	8·9	2·9	3·6	4·9	2·4	3·2	1·9

With ice cooling. The above experiment was repeated using 2 g. of drug and ice cooling throughout. The carbon tetrachloride acquired no odour of benzyl bromide and deposited no solid material. The aqueous layer now retained only 6 per cent of the added ^{14}C and gave no amine when distilled. Addition of sulphuric acid and then potassium bromide solution caused separation of an oil in the aqueous phase. Gas was not evolved until the addition of bromide was almost complete. The oil passed into the solvent layer which became deep orange in colour and soon deposited a sticky red mass (2·35 g.) which contained 90 per cent of the added ^{14}C. The aqueous layer was neutral, contained 3 per cent of the

added ^{14}C, and after basifying, gave bases on distillation equivalent to 260 ml. of N acid per mole drug. The red mass was drained on porous tile to give 1·8 g. of a yellow solid which was washed with acetone and crystallised from aqueous acetone in white prisms, m.p. 198–199° (Found: C, 38·6; H, 5·3; N, 12·9; Br, 36·6; loss at 100°, 2·2. Calc. for $C_{14}H_{23}Br_2N_4O_2$: C, 38·4; H, 5·3; N, 12·8; Br, 36·6 per cent). It was easily soluble in water, slightly soluble in ethanol but not in other common solvents. The bromine was wholly ionic in aqueous solution. There was a weak ultra-violet absorption peak at 253 mμ. With aqueous potassium iodide, it gave a white precipitate, m.p. 205°, containing 45·0 per cent iodide ion, and it gave a brown stain with Dragendorff's reagent on paper.

Dry distillation at about 200° gave benzyl bromide (44 per cent) identified by conversion to benzyltrimethylammonium bromide, m.p. 219–220° (Found: Br, 34·3. Calc. for $C_{10}H_{16}BrN$, 34·8 per cent).

Distillation of the bromide in 5N sodium hydroxide led to evolution of methylamine during 30 min., equivalent to 1·66 mole per mole $C_{14}H_{23}Br_2N_4O_2$. The aqueous residue deposited a white precipitate which crystallised from aqueous ethanol, m.p. 146–150°.

In further experiments using standard methods it was found that 1 mole drug consumed 1 mole hypobromite during bromination. During electrometric titration with 2N sulphuric acid and potassium bromide solution, added in small portions alternately at 0°, there was disappearance of 2·4 g. hydrogen ion and consumption of 2·6 atom bromine per mole drug. 2·0 atom of the total bromine used per mole drug was not ionised. During the reaction only 0·006 atom bromine per mole drug was swept out by a current of nitrogen. 0·33 mole of carbon dioxide was recovered.

DISCUSSION

The urinary elimination of BW 467C60 by two subjects after oral dosage was similar to that found in cats (Boura, Duncombe, Robson and McCoubrey, 1962). About one-fifth of the oral dose was excreted by this route during 24 hr. The fall in urinary excretion during prolonged dosage suggested that the drug might be metabolised after a period of adaptation but there was no evidence for any metabolites in cats (Boura and others, 1962). The mono-*N*-demethylated product gave about 44 per cent of the colour intensity due to BW 467C60 in the assay as described, while *N*-benzylguanidine could not be detected. Both these possible metabolites, unlike BW 467C60, could be detected on paper chromatograms by conventional spray reagents for guanidines but they could not be found by spraying chromatograms of the urines examined.

The reactions of brominated BW 467C60 proved to be an interesting chemical problem. The product decomposed at room temperature, at least partially, in a manner similar to the Hofmann degradation of *N*-bromoamides. A search for an anticipated hydrazine was not successful though a small amount of an unidentified basic oil was isolated. There are two possible *N*-bromo derivatives of BW 467C60 but it is difficult to conceive how either could absorb 2 hydrogen ions in company with 2 bromine atoms. The empirical formula of the main reaction product

isolated after treatment with acid potassium bromide at 0° (a C_{14} compound or possibly some other multiple of C_7) must arise by condensation of 2 or more molecules of BW 467C60 (C_{14}) but to obtain a C_{14} compound containing benzyl and 2 methylamine residues from 2 molecules of BW 467C60 can only be achieved by cleavage of a benzyl group (reaction with the solvent was not excluded). Since the product had no detectable biological activity in the conscious rat at a moderate dose level, investigation of the purely chemical problem was discontinued, being outside the scope of chemical pharmacology. The action of alkaline hypobromite on guanidines has not been described since 1926 when Cordier found differences in the degree of elimination of nitrogen between individual guanidines.

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THE EFFECT OF LOWERED TEMPERATURE ON THE NEUROMUSCULAR BLOCKING ACTION OF SUXAMETHONIUM ON THE RAT DIAPHRAGM

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The neuromuscular blocking action of suxamethonium on the rat isolated phrenic nerve diaphragm preparation was observed at 20 and at 37°. The effect of lowered temperature is to intensify the neuromuscular block produced by suxamethonium and to render the depolarisation characteristics of the blockade more distinct. Competitive inhibition is not apparent at 20° in contrast to the competitive features of the block which have been reported to be present at 37°.

BIGLAND, Goetzee, Maclagan and Zaimis (1958), using nerve muscle preparations *in situ* in cats and dogs showed that cooling much prolonged the action of suxamethonium without affecting the nature of the blockade no matter how long the paralysis lasted. A tetanus was always well sustained and did not antagonise the block, neostigmine was ineffective or prolonged the block and tubocurarine antagonised it. Their results from experiments on the isolated rat diaphragm were similar to those obtained in the whole animal, namely cooling greatly increased both the magnitude and duration of the blockade.

Since the depolarising action of suxamethonium is complicated by some measure of competitive inhibition in the isolated rat diaphragm at 37° (Whittaker, 1962), it seemed useful to investigate further the nature of the neuromuscular blockade at lower temperatures.

EXPERIMENTAL

Method

Bülbring's preparation was used (1946). Supramaximal rectangular pulses of 0·1 to 0·3 msec. duration were applied to the phrenic nerve at 6/min. and the muscle contractions were recorded by a spring-loaded lever. The muscle was immersed in a bath of 75 ml. capacity containing the modified Tyrode solution used in previous work (Whittaker, 1962). The fluid was aerated with 95 per cent oxygen and 5 per cent carbon dioxide at temperatures of 20° and 37° $\pm$ 0·25°. Doses are of suxamethonium bromide, tubocurarine chloride, neostigmine bromide, edrophonium bromide and potassium chloride.

RESULTS

The Effect of Temperature on the Preparation

West (1947) found that the preparation maintained the original size of contractions for a longer period if the temperature of the bath fluid was lowered from 37 to 20°. On cooling from 37° the muscle tension first increased, reaching a maximum at 25 to 30°, then declined slightly;

below 20° the speed of contraction was greatly decreased (Burgen, Dickens and Zatman, 1949). These observations have been confirmed and also it was found that the effects of temperature change were reversible; that is the described tension changes took place either when the contractions were recorded first at 20° and then after raising the temperature to 37° or when the contractions were recorded first at 37° and then after lowering the temperature to 20°. For the experiments at lower temperature, 20° was selected because, as observed by West (1947), the preparation at 20° showed little sign of fatigue even after 8 hr.

The Effect of Varying Doses of Suxamethonium at 20°

Doses in the range of 40 to 100 μg. produced an initial potentiation of the diaphragm contractions followed by the onset of a slow neuromuscular block. As the dose increased in this range the extent and duration of the potentiation was reduced and, as would be expected, the onset of neuromuscular blockade was earlier. In general, doses greater than 100 μg. produced a complete block; the time needed for complete block shortening as the dose increased. When the indirectly excited maximal twitches were completely blocked, the muscle still responded to direct stimulation.

FIG. 1. The absence of antagonism by potassium chloride of the neuromuscular block produced by suxamethonium. Rat isolated diaphragm preparation stimulated through phrenic nerve, 6/min. At S, suxamethonium added to bath fluid. In (b) potassium chloride added to bath fluid 4 min. after suxamethonium. In (c) control with potassium alone is shown. At W, preparation washed with Tyrode solution. Temp. 20°. Time 30 sec.

The Effect of Potassium Chloride on the Neuromuscular Block Produced by Suxamethonium at 20°

When suxamethonium alone was added to the bath fluid almost complete block was produced in 10 min. (Fig. 1a) and potassium chloride added 4 min. after the suxamethonium had little effect on the block (Fig. 1b). The increase in response of the diaphragm when potassium chloride was added to the bath fluid in the absence of suxamethonium is shown in Fig. 1c.

The Effect of Anticholinesterases on the Neuromuscular Block Produced by Suxamethonium at 20°

Neostigmine. The addition of suxamethonium 80 μg. alone to the bath fluid produced 84 per cent reduction in the contraction height in 12 min. (Fig. 2a). However, when neostigmine was added 4 min. after the suxamethonium the block was quickly intensified and a complete block resulted within 10 min. (Fig. 2b).

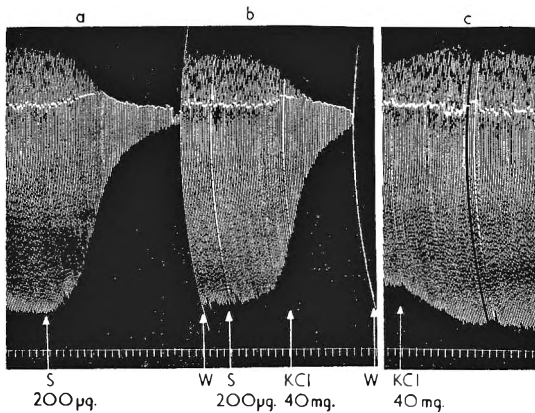
FIG. 2. The intensification by neostigmine of the neuromuscular block produced by suxamethonium. Rat isolated diaphragm preparation stimulated through phrenic nerve, 6/min. At S, suxamethonium added to the bath fluid. In (b) at N, neostigmine added to the bath fluid 4 min. after suxamethonium. At W, preparation washed with Tyrode solution. Temp. 20°. Time 30 sec.

Edrophonium. The addition of edrophonium to the bath fluid 8 min. after suxamethonium also caused intensification of the neuromuscular block,

The Effect of Tubocurarine on the Neuromuscular Block Produced by Suxamethonium at 20°

Suitable doses of suxamethonium were added to the bath fluid and followed at various time intervals by varying doses of tubocurarine. It was found that tubocurarine produced a marked though transitory antagonism of the suxamethonium block, which was then followed by a slowly progressive block in the presence of suxamethonium and tubocurarine (Fig. 3b). A further dose of tubocurarine added to the bath fluid did not antagonise the combined block but in general caused further intensification (Fig. 3b).

The Effect of Varying Doses of Suxamethonium at 37°

Varying amounts of suxamethonium were added to the bath fluid and allowed to act for 15 min. in some experiments, for 30 min. in others, or for shorter periods if a complete neuromuscular block was produced. The preparation was washed several times and allowed to recover.

Doses in the range 40 to 100 μg. when added to the bath fluid had little effect on the contractions of the diaphragm, in contrast to the initial potentiation of response before neuromuscular block produced by these doses at 20°. Doses in the range of 100 to 800 μg. occasionally produced a slight initial potentiation of contractions which was followed by a slow
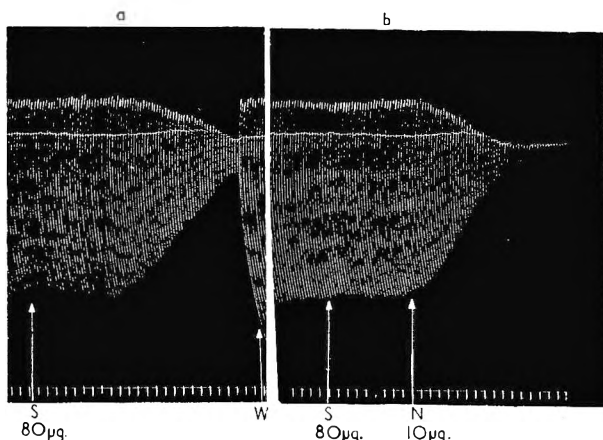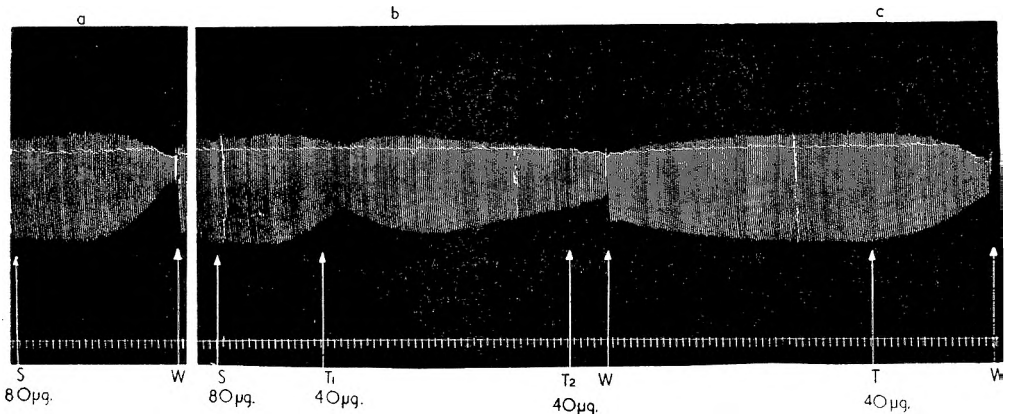

FIG. 3. The antagonism by tubocurarine of the neuromuscular block produced by suxamethonium. Rat isolated diaphragm preparation, stimulated through phrenic nerve, 6/min. At S, suxamethonium added to bath fluid. In (b) at T_1, tubocurarine added to bath fluid 8 min. after suxamethonium, antagonised the block. A subsequent dose of tubocurarine (T_2) added to bath fluid failed to produce further antagonism. (a) and (c) are control results with suxamethonium and tubocurarine alone. At W, preparation washed with Tyrode solution. Temp. 20°. Time 30 sec.

block. In most experiments however the initial potentiation of the response was absent, the neuromuscular block showed an initial fairly sharp onset, then progressed to a steady level and later declined slowly (Fig. 4). For complete block doses greater than 800 μg. were required.

DISCUSSION

The rat diaphragm preparation is more sensitive to suxamethonium at 20° than at 37°. This is in accordance with the findings of Bigland and others (1958). Moreover the neuromuscular block produced by suxamethonium at 20° appears to be due to end-plate depolarisation, as indicated by an initial stimulation before the onset of the block, the lack of effect of potassium on the block, intensification by the anticholinesterases, neostigmine and edrophonium and antagonism of the block by tubocurarine.

Whittaker (1962) reported that at 37° there were competitive features in the neuromuscular block produced by suxamethonium in the rat diaphragm preparation, the block being antagonised by potassium and intensified by tubocurarine.

Several workers have shown that suxamethonium is hydrolysed mainly by plasma cholinesterase in two stages (i) fairly rapidly to succinylmonocholine and choline and (ii) much more slowly to succinic acid and choline (V. P. Whittaker and Wijesundera, 1952; Tsuji, Foldes and Rhodes, Jr.,

1955). Also the breakdown of suxamethonium *in vitro* by both true and pseudocholinesterases has been reported (Low and Tammelin, 1951). Stovner (1958) studied the action of succinylmonocholine and succinyl-dicholine in nerve-diaphragm preparations of rats and kittens and in nerve muscle preparations *in situ* in rabbits and cats, and concluded that the neuromuscular block produced by succinylmonocholine was

S   W
600µg.
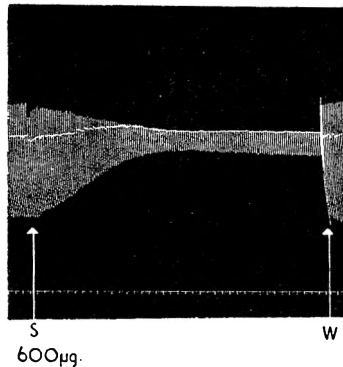
FIG. 4. The neuromuscular block produced by suxamethonium (S) at 37°. Rat isolated diaphragm preparation stimulated through phrenic nerve, 6/min. Initial potentiation of response is absent. The neuromuscular block consists of an initial phase of fairly sharp onset and a prolonged phase at a steady level. At W, preparation washed with Tyrode solution. Time 30 sec.

more competitive in nature than the block produced by succinyldicholine. Therefore Whittaker (1962) suggested that the competitive features of the suxamethonium block in the rat diaphragm preparation at 37° might be related to the enzymatic hydrolysis of suxamethonium and the formation of succinylmonocholine. The increased effect of suxamethonium at 20° and the absence of competitive features from the neuromuscular block may be due partly to the reduced cholinesterase activity at the lower temperature. However, the effects cannot be due solely to inactivation of the enzyme since Bigland and others (1958), have shown that cooling affects a neuromuscular block produced by decamethonium more, or at least as much as, a block produced by suxamethonium and it is known that decamethonium is not destroyed enzymatically in the same manner as suxamethonium (Zaimis, 1950).

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THE BACTERIOSTATIC ACTIONS OF TETRACYCLINE AND OXYTETRACYCLINE

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The bacteriostatic actions of tetracycline and oxytetracycline on
cultures of *A. aerogenes* in glucose-mineral salt media have been
studied. Both antibiotics show two modes of action, one of which is
non-operative under aerobic conditions and is ascribed to an inter-
ference with the disposal of hydrogen in unaerated cultures. The
other mode of action can be accounted for by an interference with the
synthesis of protein. Quantitative measurements of the dependence
of the degree of inhibition on the pH and the concentration of mag-
nesium ions are in accord with attributing inhibitory power solely to the
non-ionic molecular forms of the antibiotics. The possible contribu-
tions of the two modes of action to the cytotoxic action are discussed.

IT has been suggested variously (Eagle and Saz, 1955) that tetracyclines
affect oxidation and fermentation as well as derange the synthesis of
protein in cells, and, since they have an affinity for inorganic cations, that
they may interfere with cellular and enzymatic processes which require
such ions. Many of the investigations which have led to these suggestions
have been made with much larger concentrations than those obtained
clinically (Goldberg, 1959a) which in the case of tetracycline itself is 1 to
2 μM in the blood (Welch, Lewis, Staff and Wright, 1957), and many
were made with isolated parts of bacterial metabolism. Thus, the cyto-
toxic action of tetracyclines remains obscure.

Recently it has been shown that the ratio of the mean generation time
of an inhibited culture to the mean generation time of a corresponding
uninhibited culture, which has been termed the "index ratio", is a sensitive
and reproducible measure of the amount of inhibition caused by a small
concentration of an inhibitor in cultures of a thoroughly stabilised
organism, and that abrupt changes in the plot of index ratio against
concentration of inhibitor indicate changes from one mode of inhibition
to another (Harris and Morrison, 1961). This means of measuring
inhibition is now used to show that removal of inorganic cations is not a
likely mode of action of tetracycline or oxytetracycline, to establish that
only one of the possible molecular and ionic forms (Goldberg, 1959b)
has inhibitory powers, and to show that both tetracyclines have two modes
of action at concentrations that are realisable clinically.

METHODS AND MATERIALS

Organism and Media

Since the method requires a thoroughly stabilised organism which
possesses the maximum synthesising abilities two sub-strains of the
Aerobacter aerogenes utilised by Dagley, Dawes and Morrison (1951),

which had been maintained by regular sub-culturing (unaerated and aerated respectively) in synthetic medium for eight years, were selected. Both sub-strains showed the same reactions to various sugars in Bacto-purple broth, to Bacto Simmons Citrate Agar, MR-VP medium, and Bacto-SIM medium (except for motility in the latter test) as described for *A. aerogenes* in the 9th edition of the Difco manual. The basic medium contained 5·4 g. KH_2PO_4, 12 g. glucose, 1·8 g. $(NH_4)_2SO_4$ and 0·0203 g. $MgSO_4·7H_2O$ (all Analar quality) per litre of demineralised water of resistance greater than 2×10^6 ohms per cm., and was adjusted to the required pH (usually 7·00) with NaOH. The concentration of magnesium in this medium is 0·082 mM which is one tenth of the lower limit of the concentration (2 to 4 mg./100 ml.) in human blood (Bell, Davidson and Scarborough, 1950), but twice the minimum concentration required to maintain the normal growth characteristics of the test organism. When higher concentrations of magnesium or the presence of other materials were required, part of the water in the basic medium was replaced by a suitable solution. All inocula (usually 0·10 ml. to 10·0 ml. of medium) were taken after growth had stopped, from parent cultures containing only sufficient glucose to permit two-thirds of the normal extent of growth; a procedure which ensures reproducible results and negligible lags before growth. The extents of growth were obtained by measurements of optical density of killed samples using a "Unicam S.P.400" spectrophotometer at 583 mμ calibrated in mg. dry cells per ml. Rates of growth (mean generation times) were obtained graphically, were reproducible to ± 3 per cent, and could be determined from data obtained from a growing culture before its pH had fallen by 0·2 units. Cultures in unaerated basic media in 7 in. by 1 in. tubes of the sub-strain accustomed to the conditions, have a mean generation time of 38 ± 1 min., and cultures of the other sub-strain in basic media aerated with sterile, filtered air containing an adequate concentration of CO_2 (Morrison, Griffiths and Harris, 1955), have a mean generation time of 32 ± 1 min.

Antibiotics

Tetracycline as the hydrochloride "Tetracyn" and oxytetracycline as "Terramycin" were donated generously by Messrs. Pfizer Ltd. Aqueous solutions of each, and solutions containing magnesium ions, were titrated potentiometrically with aqueous NaOH using the conditions described by Albert and Rees (1956). The dissociation constants K_1, K_2 and K_3, and the stability constants K_s for the complexes with magnesium, calculated conventionally, are given with corresponding values from the literature in Table I.

Since each compound has four ionic or molecular forms $TH_3^+Cl^-$ will be used to represent tetracyline hydrochloride and $\emptyset H_3^+Cl^-$ to represent oxytetracycline hydrochloride.
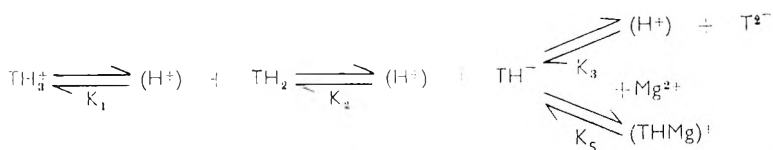
The potentiometric titration curves in the presence and absence of magnesium are concurrent initially when, by calculation from the dissociation constants, the tetracycline compound is present as a mixture of TH_3^+ and TH_2, or $\emptyset H_3^+$ and $\emptyset H_2$, but deviate when it is present as a
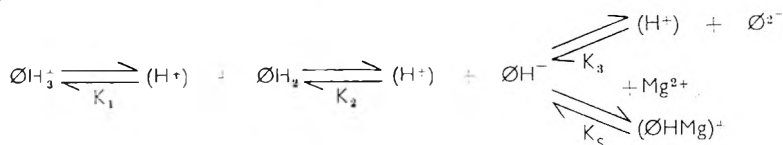
TABLE I

IIONISATION CONSTANTS (K_1, K_2, K_3) OF TETRACYCLINE AND OXYTETRACYCLINE HYDRO-CHLORIDES AND STABILITY CONSTANTS $K(s)$ OF THE COMPLEXES WITH MAGNESIUM

Source of information	Tetracycline				Oxytetracycline			
	pK_1	pK_2	pK_3	pK_S	pK_1	pK_2	pK_3	pK_S
Present work	3·45	7·92	9·81	4·16–4·29	3·46	7·76	9·70	3·96
Albert (1953) or Albert and Rees (1956)	3·35	7·82	9·57		3·10	7·26	9·11	3·80
Stephens and others (1956)	3·30	7·68	9·69		3·27	7·32	9·11	
Regna and others (1951)					3·45	7·55	9·24	

mixture of TH_2 and TH^-, or $ØH_2$ and $ØH^-$. Clearly Mg^{2+} combines with the ionic forms TH^- and $ØH^-$. Between pH 7·00 and 7·80 the forms of the compounds present in significant concentrations are TH_2 and TH^-, and $ØH_2$ and $ØH^-$. The relationship between the molecular and ionic forms are:

$$TH_3^+ \underset{K_1}{\rightleftharpoons} (H^+) + TH_2 \underset{K_2}{\rightleftharpoons} (H^+) + TH^- \underset{K_3}{\rightleftharpoons} \begin{array}{c} (H^+) + T^{2-} \\ +Mg^{2+} \\ \underset{K_S}{\rightleftharpoons} (THMg)^+ \end{array}$$

and

$$ØH_3^+ \underset{K_1}{\rightleftharpoons} (H^+) + ØH_2 \underset{K_2}{\rightleftharpoons} (H^+) + ØH^- \underset{K_3}{\rightleftharpoons} \begin{array}{c} (H^+) + Ø^{2-} \\ +Mg^{2+} \\ \underset{K_S}{\rightleftharpoons} (ØHMg)^+ \end{array}$$

RESULTS

Extent of Inhibition and Concentration of Antibiotic

In basic media. Increasing concentrations of either antibiotic cause a progressive increase of mean generation time of both aerated and un-aerated cultures. The results of plotting the calculated index ratios against the molar concentrations of antibiotic are shown in Fig. 1.

After a threshold concentration of between 0 and 0·035 μM tetracycline, in aerated cultures, and in unaerated cultures up to 0·2 μM, the index ratios are found to plot together (Mode I tetracycline). At concentrations greater than 0·2 μM in unaerated cultures tetracycline inhibits more severely (Mode II tetracycline). Inhibition by oxytetracycline of aerated cultures (Mode I oxytetracycline) resembles closely Mode I tetracycline, but the inhibition of unaerated cultures is more severe at all concentrations (Mode II oxytetracycline).

In enriched media. Adding to the basic medium the product of an inhibited reaction, provided that it can penetrate the cells, must eliminate the inhibited reaction's control of the rate of growth (Harris and Morrison, 1961). The following materials were used to enrich the medium: bacteriological peptone (Difco), casein hydrolysate (prepared by D. E. Griffiths in this laboratory and consisting solely of amino-acids), individual

amino-acids (Messrs. Light or B.D.H.) and yeast-extract (Difco), all of which can replace the glucose or the ammonium salt, or both, in basic medium without preventing growth of the organism. None of these materials affected the degree of inhibition of aerated cultures by 0 to
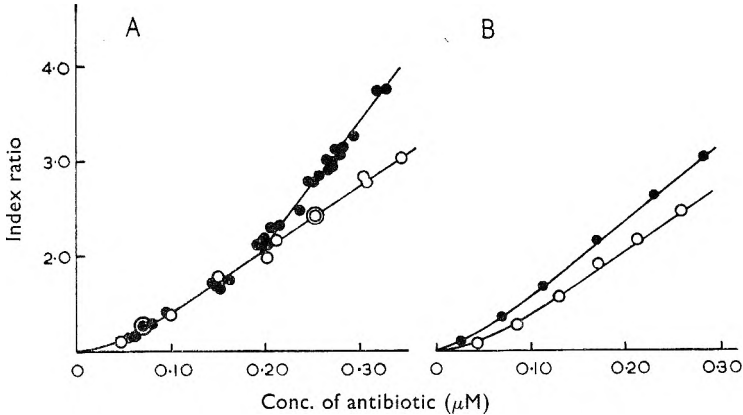
FIG. 1. Inhibition of cultures of *A. aerogenes* by tetracycline and oxytetracycline. Index ratio is (the mean generation time of the inhibited culture) divided by (the mean generation time of the uninhibited culture). A, tetracycline; B, oxytetracycline; ●, unaerated cultures; ○, aerated cultures.

0·35 μM tetracycline or oxytetracycline, nor did the peptone, casein hydrolysate nor any individual amino-acid affect the inhibition of unaerated cultures. The affect of yeast-extract at concentrations of 0·2 per cent w/v or greater in unaerated cultures are compared with the inhibitions of plain unaerated and aerated cultures in Table II.

In both examples the inhibition of unaerated cultures containing yeast-extract is indistinguishable from the inhibition of aerated cultures in basic

TABLE II

EFFECT OF 0·2 PER CENT W/V YEAST-EXTRACT ON INHIBITION BY TETRACYCLINE AND OXYTETRACYCLINE

Concentration of antibiotic μM	Index ratio ± 0·08		
	A Unaerated basic medium	B A plus yeast-extract	C Aerated basic medium
Tetracycline			
0·051	1·12	1·12	1·14
0·103	1·40	1·37	1·40
0·154	1·68	1·67	1·75
0·206	2·15	2·03	2·10
0·214	2·22	2·07	2·16
0·220	2·30	2·05	2·20
0·248	2·70	2·31	2·40
0·258	2·80	2·36	2·46
0·275	3·10	2·59	2·58
0·283	3·05	2·61	2·64
0·303	3·47	2·83	2·77
Oxytetracycline			
1·02	1·56	1·40	1·36
2·26	2·62	2·40	2·22
3·19	3·30	2·88	2·92

medium. Thus yeast-extract provides an "antidote" to both Mode II tetracycline and to Mode II oxytetracycline.

The nucleotide fraction of the yeast-extract isolated by the method of Clark, Dounce and Stotz (1949) did not reproduce the effect of the yeast-extract on inhibition by tetracycline. In further tests the dicarboxylic acids oxalo-acetic, fumaric and succinic were used: none decreased the inhibition of aerated cultures by tetracycline and only fumaric at 0·4 per cent w/v had a detectable effect on inhibition of unaerated cultures but this is not sufficient to be an elimination of Mode II tetracycline (Table III).

TABLE III

EFFECT OF 0·4 PER CENT W/V FUMARIC ACID ON INHIBITION OF UNAERATED CULTURES BY TETRACYCLINE

Concentration of tetracycline, μM	Index ratio ±0·08		
	A Unaerated basic medium	B A plus fumarate	C Aerated basic medium
0·214	2·22	2·07	2·16
0·299	3·44	3·20	2·75
0·320	3·72	3·47	2·89

These results suggest that the efficiency of the yeast-extract is due to its content of reducible substance(s) and not to its provision of co-enzymes I or II.

Extent of Inhibition and the Concentration of Magnesium

Tetracycline in unaerated cultures. The index ratios were determined for cultures containing varying concentrations of magnesium (82 μM to 6·56 mM) and tetracycline (0 to 0·3 μM). In all experiments the angles, measured from the horizontal ordinate, of the plots corresponding to those in Fig. 1, decrease progressively as the concentration of magnesium increases. This indicates a decreasing degree of inhibition. The results for unaerated cultures inhibited by tetracycline are shown in Fig. 2.

Since magnesium ions associate with sulphate and phosphate ions the concentration of free magnesium ions must be calculated before the experimental data can be examined to ascertain whether the inhibitors operate by removing magnesium ions or whether magnesium ions decrease the inhibition by removing molecules or ions of the inhibitor.

The medium contains 0·0397 F KH_2PO_4 and 0·0136 F $(NH_4)_2SO_4$ and both phosphate and sulphate associate with Mg^{2+} ions.

Since pK_2 of $H_2PO_4^-$ is 7·2 at 25° (Robinson and Stokes, 1959) at pH = 7·00, $[HPO_4^{2-}] = 0·0153$ and $[HPO_4^{2-}]/[H_2PO_4^-] = 0·63$ in the presence of Mg^{2+} ions,

$$[HPO_4^{2-}] = C_L - [H_2PO_4^-] - [MgHPO_4]$$

where C_L = the total concentration of phosphate = 0·0379, and assuming that Mg^{2+} and $H_2PO_4^-$ do not associate,

$$\therefore [C_L - [H_2PO_4^-] - [MgHPO_4]]/[H_2PO_4^-] = 0·63 \quad .. \quad .. \quad (1)$$

Since K of $HSO_4^- = 0.01$ (Robinson and Stokes, 1959), $[HSO_4^-]$ is negligible.

Now (Dunsmore and James, 1951):

$$K_{s(MgSO_4)} = [Mg^{2+}] [SO^{2-}]/[MgSO_4] = 0.006$$

and (Redish and Kibrick, 1940)

$$K_{s(MgHPO_4)} = [Mg^{2+}] [HPO_4^{2-}]/[MgHPO_4] = 0.003$$

Hence

$$[MgHPO_4] = [C_M - [MgHPO_4] - [MgSO_4]] [HPO^{2-}]/0.003 \qquad (2)$$

and

$$[MgSO_4] = [C_M - [MgHPO_4] - [MgSO_4]]$$
$$[0.0138 - [MgSO_4]]/0.006 \qquad (3)$$

where C_M = the total concentration of magnesium, which in the basic medium is 82 μM.
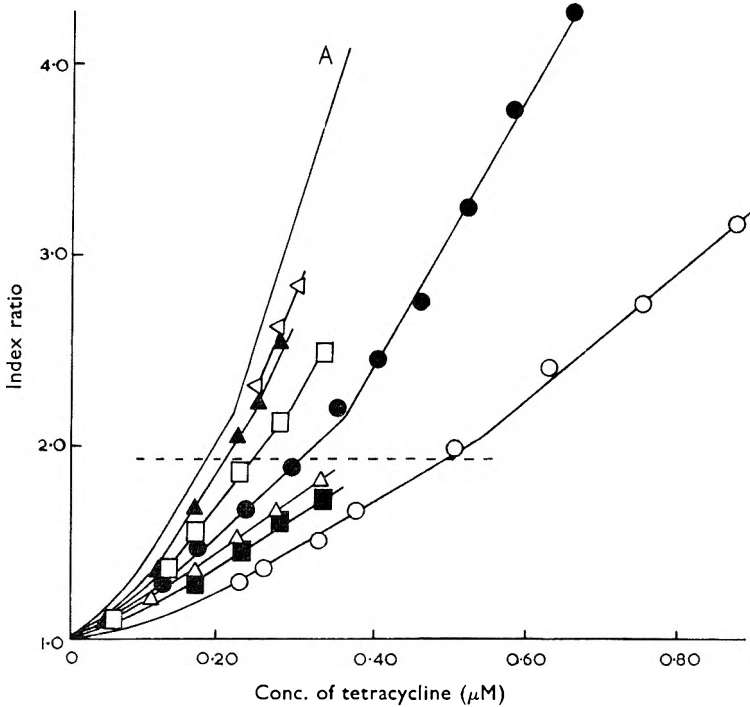
FIG. 2. Magnesium concentration and inhibition of unaerated cultures of *A. aerogenes* by tetracycline. Concentration of magnesium (mM): A, from Fig. 1, 0.082; ◁, 0.656; ▲, 0.820; □, 1.64; ●, 2.46; △, 3.28; ▨, 4.10; ○, 6.56.

Ignoring $[MgSO_4]$ in the right hand side of equation (2), from (1) and (2) $[MgHPO_4] \doteq 68 \mu$M and $[Mg^{2+}] \doteq 1.4 \mu$M. Putting these rough values into (3) $[MgSO_4] \doteq 32 \mu$M. Since $C_M = [Mg^{2+}] + [MgHPO_4] + [MgSO_4]$ the rough value for $[Mg^{2+}]$ is too high, but trial and error with

lower values calculating [MgHPO$_4$] and [MgSO$_4$] to test in the equation for C_M establishes that

$$[Mg^{2+}] = 9.8 \ \mu M \ \text{and} \ \therefore \ [Mg^{2+}]/C_M = 0.120$$

Similarly when $C_M = 6.56$ mM, $[Mg^{2+}]/C_M = 0.132$.

Thus in all the media at pH 7.00 only $\frac{1}{8}$th of the magnesium was present as free Mg^{2+} ions after allowing for association with phosphate and sulphate. In the present experiments the concentrations of inhibitors varied from 0 to 1 μM whereas the concentration of free Mg^{2+} ions varied from 10.2 to 86.6 μM. Association of Mg^{2+} and tetracycline or oxytetracycline cannot have had a significant effect on the concentration of free Mg^{2+} ions and hence neither antibiotic can be exerting an inhibitory action by decreasing the concentration of free Mg^{2+} ions. Since the values of K_s for the interactions of other cations with the two tetracyclines are similar to that for the magnesium complexes (Albert and Rees, 1956) and other cations must be assumed to associate with sulphate and phosphate, it is unlikely that the inhibitory actions can be accounted for by a decrease in the concentration of any other cation in the presence of the antibiotics.

If the higher concentrations of magnesium decrease the inhibition by decreasing the concentration of free antibiotic then points having the same index ratio on the experimental plots, one for each total concentration of magnesium, in Fig. 2 (for example intercepts with the horizontal broken line) must correspond to cases in which the concentration of free antibiotic is the same. It is thus possible to obtain a number of series of paired concentrations of magnesium and of an antibiotic which yield the same concentration of free antibiotic. The plot of concentration of magnesium against concentration of antibiotic for each such series is a shallow curve which can be extrapolated shortly to cut the concentration of antibiotic ordinate. Each intercept gives the concentration of antibiotic which would cause the particular degree of inhibition in the absence of association with magnesium, and hence the concentration of free antibiotic responsible for the particular value of the index ratio for the series.

Since at pH 7.00 the free tetracycline is present solely as TH_2 and TH^-, and the dissociation constant K_2 is known, the concentrations of TH_2 and TH^- can be calculated from the concentration of free tetracycline. It is thus possible to obtain corresponding values of [TH_2], [TH^-], [bound tetracycline] (total concentration of tetracycline $-$ [TH_2] $-$ [TH^-]) and [free Mg^{2+}] (calculated from total concentration of magnesium as before) provided that the total concentration of magnesium is sufficiently large to ensure that [Mg^{2+} (free)] is not affected significantly by combination of magnesium and tetracycline.

When the experimental data used for Fig. 2 are processed in this way, for any one particular index ratio log [Mg^{2+}] plots linearly with a slope of unity against log [bound tetracycline], and log [TH^-] also plots linearly with a slope of unity against the same ordinate except when the concentration of tetracycline is relatively high, then the slope decreases. This indicates that the complex between magnesium and tetracycline is

normally $MgTH^+$ but possibly some $Mg(TH)_2$ is obtained at the higher concentrations of tetracycline. The previously determined [bound tetracycline] may be expressed as $[MgTH^+]$.

Since the stability constant, K_s, for $MgTH^+$ is given by

$$\log K_s = \log [MgTH^+] - \log [Mg^{2+}] - \log [TH^-]$$

log K_s can be calculated from the results of the experiments with bacteria. This is done in Tables IV and V.

TABLE IV

CALCULATION OF LOG K_8 FOR THE MAGNESIUM-TETRACYCLINE COMPLEX. PART 1

Index Ratio	T_f	$\dfrac{[Mgt]}{[Mg^{2+}]}$	82·0 10·25	164 20·50	246 30·75	328 41·00	410 51·25	656 82·00
1·20	0·64–	T_t	0·78	0·85	1·00	1·16	1·35	1·66
	0·69	T_b	0·09–	0·16–	0·31–	0·47–	0·66–	0·97–
			0·14	0·21	0·36	0·52	0·71	1·02
1·40	0·97–	T_t	1·17	1·29	1·61	1·79	2·13	2·58
	1·00	T_b	0·17–	0·29–	0·61–	0·79–	1·13–	1·58–
			0·20	0·32	0·64	0·82	1·16	1·61
1·60	1·29	T_t	1·50	1·72	2·14	2·54	2·90	3·40
		T_b	0·21	0·43	0·85	1·25	1·61	2·11
1·80	1·58	T_t	1·83	2·13	2·68	3·22	3·65	4·18
		T_b	0·25	0·55	1·10	1·64	2·07	2·60
2·00	1·85	T_t	2·14	2·56	3·22			4·97
		T_b	0·29	0·71	1·37			3·12
2·20	2·09	T_t	2·42	2·92	3·64			5·64
		T_b	0·33	0·83	1·55			3·55
2·40	2·23	T_t	2·64	3·18	3·94			6·30
		T_b	0·41	0·95	1·71			4·07
2·60	2·38	T_t			4·23			6·94
		T_b			1·85			4·56
2·80	2·54	T_t			4·52			7·57
		T_b			1·98			5·03
3·00	2·69	T_t			4·81			8·19
		T_b			2·12			5·50
3·20	2·83	T_t			5·09			8·80
		T_b			2·26			5·97

T_t = Total concentration of tetracycline ($M \times 10^7$)
T_f = Concentration of free tetracycline ($M \times 10^7$), $T_b = T_t - T_f$.
$[Mgt]$ = Total concentration of magnesium ($M \times 10)^2$
$[Mg^{2+}]$ = Concentration of Mg^{2+} ions ($M \times 10^5$)

Thus the theory of a non-inhibitory complex of magnesium and tetracycline requires the complex to be $MgTH^+$ with a stability constant of antilog (4·07 to 4·38) in order to have a quantitative fit to the experimental evidence. Potentiometric titrations require the formula to be $MgTH^+$ and the stability constant to be antilog (4·16 to 4·29) (Table I).

Extent of inhibition and pH

Tetracycline in unaerated cultures. The overall appearance of the graphical representations corresponding to Fig. 1 of the results of experiments in media having an initial value of pH other than 7·00, is unchanged, but decreasing the pH increases the slopes of the linear portions of the figure and increasing the pH decreases them. The index ratios for a number of concentrations of tetracycline in unaerated cultures in basic media initially at pH 7·00 and 7·80 are compared in Table VI.

Since at pH 7·00 and 7·80 the tetracycline is present as TH_2, TH^- and $MgTH^+$, and it has been concluded that the latter is non-inhibitory, both modes of action must be due to TH_2 and/or TH^-. At higher pH

TABLE V

CALCULATION OF LOG K_S FOR THE MAGNESIUM-TETRACYCLINE COMPLEX. PART 2

log [TH⁻]	1·01		1·31		1·49		1·62		1·71		1·92	
log [Mg²⁺] → log [TH⁻]	log T_b	log K_s	log T_b	log K_s	log T_b	log K_s	log T_b	log K_s	log T_b	log K_s	log T_b	log K_s
9·87 or	9·95	4·07	8·20	4·07	8·49	4·13	8·67	4·18	8·82	4·24	8·99	4·20
9·84	8·15	4·31	8·32	4·17	8·56	4·23	8·72	4·26	8·85	4·30	7·01	4·25
8·03 or	8·23	4·19	8·46	4·12	8·79	4·27	8·90	4·25	7·05	4·31	7·20	4·25
8·02	8·30	4·27	8·51	4·18	8·81	4·30	8·91	4·27	7·06	4·33	7·21	4·27
8·14	8·32	4·17	8·63	4·18	8·93	4·30	7·10	4·34	7·21	4·36	7·32	4·25
8·23	8·40	4·16	8·74	4·20	7·04	4·32	7·21	4·36	7·32	4·38	7·42	4·27
8·30	8·46	4·15	8·85	4·24	7·14	4·35					7·49	4·27
8·35	8·52	4·16	8·92	4·26	7·19	4·35					7·55	4·28
8·38	8·61	4·22	8·98	4·29	7·23	4·36					7·61	4·31
8·41					7·27	4·37					7·66	4·33
8·43					7·30	4·38					7·70	4·35
8·46					7·33	4·36					7·74	4·36
8·48					7·35	4·38					7·78	4·38

log K_s = log T_b − log [Mg²⁺] − log [TH⁻] = 4·07 to 4·38

the [TH⁻] due to a particular concentration of tetracycline is larger, [TH₂] is smaller and the inhibitory power is less. This suggests that TH₂ is the inhibitor, in which case the degree of inhibition should reflect [TH₂] irrespective of the pH.

From the equations

$$K_S = [MgTH^+]/[Mg^{2+}] [TH^-]$$

$$K_2 = [H^+] [TH^-]/[TH_2]$$

$$T_t = [TH^-] + [TH_2] + [MgTH^+]$$

where T_t = the total concentration of tetracycline,

at pH 7·00 $[TH_2] = T_t (8·32/9·53)$

at pH 7·80 $[TH_2] = T_t (1·32/2·53)$

In Fig. 3A the extent of inhibition in media initially at pH 7·00 and at pH 7·80 is plotted against T_t (broken lines) and against $[TH_2]$ (solid line). Clearly the experimental data fit the theory that of all the molecular and ionic forms only TH_2 is inhibitory.

TABLE VI

EFFECT OF pH ON INHIBITION BY TETRACYCLINE

Concentration of tetracycline (μM)..	0	0·060	0·120	0·180	0·240	0·360	0·480
Index ratio at pH 7·00	1·00	1·17	1·52	1·94	2·58	4·18	
±0·08 at pH 7·80	1·00	1·12	1·30	1·52	1·82	2·62	3·55

Extent of inhibition by oxytetracycline and its dependence on concentration of magnesium and pH. Measurements of inhibition by oxytetracycline of unaerated cultures in media containing varying concentrations of magnesium at initially pH 7·00 and in basic media at initially pH 7·80, are collated in Tables VII and VIII. Increasing the pH or the concentration of magnesium decreases the slopes of the linear portions of the diagrams corresponding to Fig. 1b, and hence attributing inhibition to the

molecular form of oxytetracycline, $\emptyset H_2$, should be tested quantitatively. At pH 7·00 and pH 7·80 the significantly effective equilibria are

$\emptyset H_2 = \emptyset H^- + (H^+)$ for which $K_2 = [H^+] [\emptyset H^-]/[\emptyset H_2]$

$\emptyset H^- + Mg^{2+} = Mg\emptyset H^+ \qquad K_s = [Mg\emptyset H^+]/[\emptyset H^-] [Mg^{2+}]$
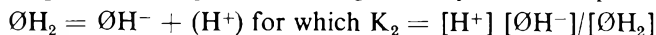
Since $\emptyset_t = [\emptyset H^-] + [\emptyset H_2] + [Mg\emptyset H^+] =$ the total concentration of oxytetracycline,

$$[\emptyset H_2] = \emptyset_t/[1 + K_2[1 + K_s[Mg^{2+}]]/[H^+]]$$

Since $[Mg^{2+}]$ can be calculated from the total concentration as previously, $[\emptyset H_2]$ for each value of $\emptyset_t$ in Tables VII and VIII can be calculated, and
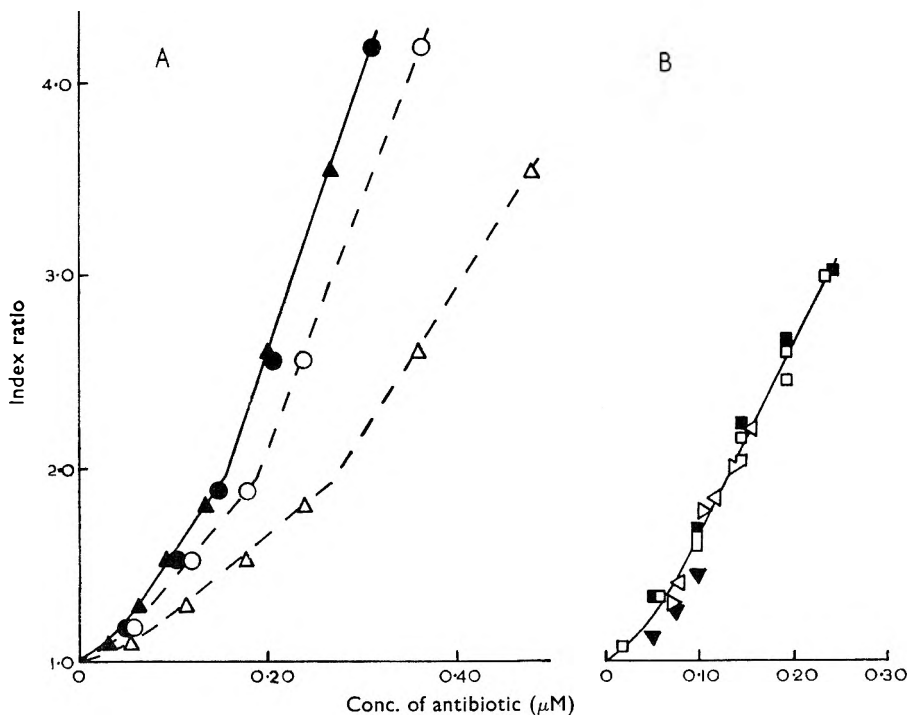

FIG. 3.   Inhibition of *A. aerogenes* as a function of the concentrations of the molecular forms of tetracycline and oxytetracycline.   Concentrations of molecular tetracycline (TH_2) and of molecular oxytetracycline ($\emptyset H_2$) are calculated using the concentrations of magnesium, sulphate and phosphate, and the initial pH of the unaerated cultures.

	tetracycline (A)		oxytetracycline (B)				
Concentration of magnesium (mM)	0·082	0·082	0·082	0·82	1·64	4·96	0·082
Initial pH	7·00	7·80	7·00	7·00	7·00	7·00	7·80
Symbol	●	▲	□	◁	▷	▼	▨

The broken lines, —○—, and —△—, show the plots resulting from using the total concentrations of tetracycline for the cultures initially at pH 7·00 and 7·80, respectively.

used as the ordinate in plots of index ratio against concentration of inhibitor.   Fig. 3B shows that the result is a single graph and hence attributing inhibitory power to only $\emptyset H_2$ is in quantitative agreement with the experimental results.

Inhibition in aerated cultures. The experimental results for tetracycline and oxytetracycline inhibiting aerated cultures examined as in the previous paragraph also are in agreement with TH_2 and $\varnothing H_2$ being the inhibitory forms of the substances.

TABLE VII

AFFECT OF MAGNESIUM CONCENTRATION ON INHIBITION OF UNAERATED CULTURES BY OXYTETRACYCLINE

Concentration of oxytetracycline μM	Index ratio ±0·08			
	Concentration of magnesium (mM)			
	0·082	0·820	1·640	4·940
0·107	1·60	1·40	1·30	1·11
0·157	2·02	1·85	1·57	1·25
0·210	1·45	2·23	2·00	1·43

TABLE VIII

EFFECT OF pH ON INHIBITION OF UNAERATED CULTURES BY OXYTETRACYCLINE

Concentration of tetracycline (μM)	0	0·023	0·068	0·113	0·169	0·226	0·283	0·339	0·452	0·565
Index ratio at pH 7·00	1·00	1·08	1·34	1·66	2·16	2·61	3·02			
±0·08 at pH 7·80	1·00			1·34		1·68		2·24	2·68	3·02

Nature of the Modes of Action

Mode II. With both antibiotics the comparison of the experimental evidence from cultures in basic and in enriched media indicates that mode II is concerned with hydrogen-transfer processes, possibly being inhibition of the production of a hydrogen acceptor required under unaerated conditions.

The transfer of hydrogen in an active bacterial system is effected by a chain of enzyme controlled reactions:

Initial source of hydrogen

Final acceptor
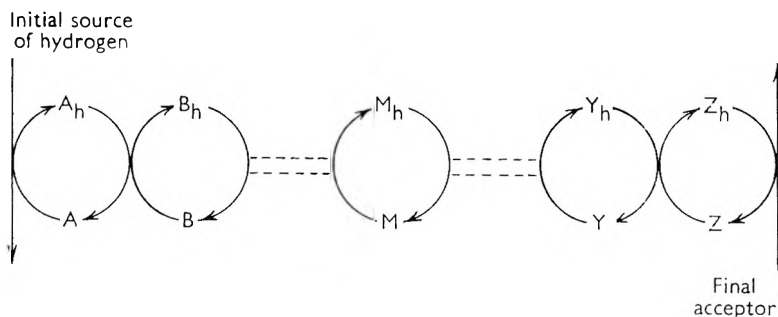
where A_h, B_h etc. are the reduced forms of A, B, etc., respectively. If the enzymic mechanism of such a chain is put in contact with the source of hydrogen, then initially there are no intermediates such as B, M or Y present. The rate of formation of any intermediate is a net rate: it is

being formed from its precursor and being consumed to form its successor, i.e., dS_M/dt = a function, f, of S_L,S_M — a function, f', of S_M, S_N

$$= f(S_L,S_M) - f'(S_M,S_N)$$

where S_L is the concentration of the precursor, S_M the concentration of intermediate itself, and S_N the concentration of its successor.

The first term increases as S_L increases but decreases as S_M increases and the second increases as S_M increases, but decreases as S_N increases. Thus the net rate of production of the intermediate rises to a maximum and then decreases until $f(S_L,S_M) = f'(S_M,S_N)$ and the steady state is attained with an overall transfer rate for hydrogen of the value of $f(S_L,S_M)$ when $dS_M/dt = 0$. The consequence of this is that a plot of concentration
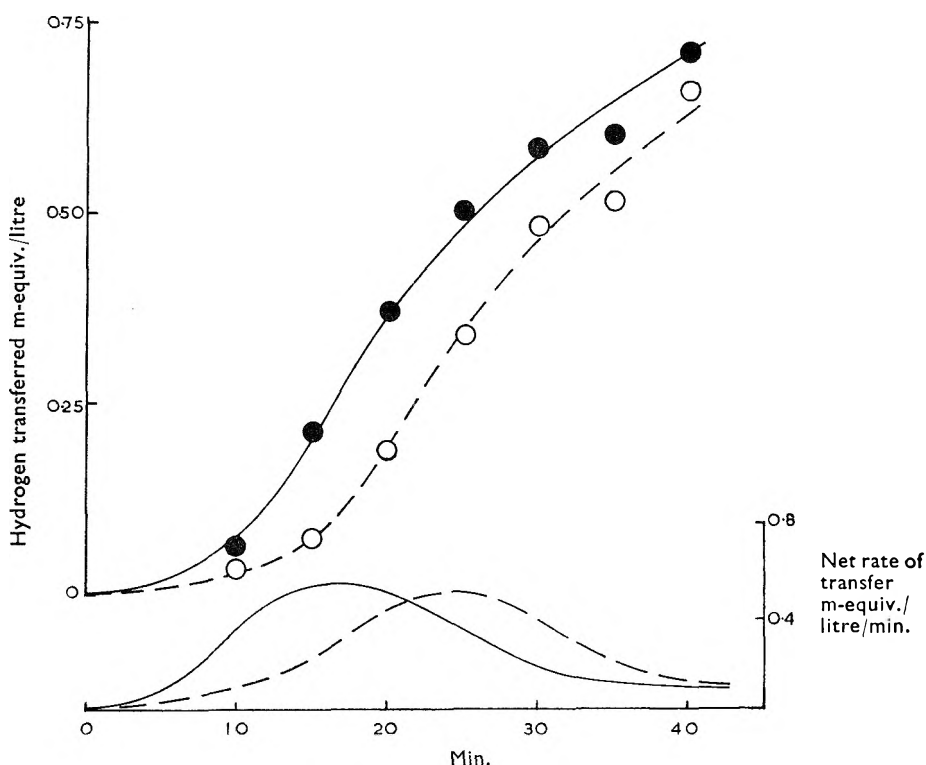
FIG. 4. Effect of tetracycline on the reduction of triphenyltetrazolium ions by a suspension of *A. aerogenes* (0·15 g. (dry) cells per litre). Concentration of tetracycline (μM): $\bigcirc$, 0; $\bullet$, 0·30.

of an intermediate against time is a sigmoid curve. The steady state is approached asymptotically and it is not possible to measure the time for it to be reached, but the time at which the maximum net rate of production of the intermediate is reached, is readily determinable as the time of inflexion of the sigmoid curve of concentration. These phenomena are illustrated by the experimental results shown in Fig. 4.

When a colourless substance which is reducible by an intermediate, (say) M_h, to a coloured substance, is added, it forms a non-enzymic branch to the system of which the coloured substance is the end-product. The rate of production of colour is dependent on the rate of production of M_h and hence will rise initially to a maximum and then decrease to a constant value when the enlarged system is in its steady state. This eventual constant rate of production of colour will absorb a definite fraction of the rate of supply of hydrogen to the system and be linearly related to the rate of production of the normal end-product. The time of maximum rate of development of colour is given by the inflexion in the curve of depth of colour against time.

The introduction of an inhibitor of a step preceeding M_h decreases $f(S_L, S_M)$, and of an inhibitor of a step succeeding M_h decreases $f'(S_M, S_N)$. In the former case the time needed for the development of the maximum rate is increased, and in the latter it is decreased. Thus comparison of these times for the inhibited and uninhibited systems indicates whether the inhibition preceeds or succeeds the point of interception of hydrogen by the leuco-compound.

Triphenyltetrazolium ions were reduced at $37°$ to formazan by suspensions of cells (0·15 g. (dry) cells per litre) in 12 g. glucose, 5·4 g. KH_2PO and 0·0203 g. $MgSO_4 \cdot 7H_2O$ per litre adjusted to pH 7·0, and in medium of the same composition except that 0·3 μmole/litre of tetracycline had been added. This reagent is reduced probably by one of the flavoproteins (Brodie and Gots, 1951; Somerson and Morton, 1953). The cells were obtained from fully grown cultures (unaerated) in which growth had been limited simultaneously by exhaustion of the glucose and ammonium salt originally present, and were resuspended directly in the test media. Cells prepared in this way are potentially highly active but in the absence of a source of hydrogen do not produce a detectable amount of formazan in 1 hr. Well washed cells with the source of hydrogen reduce triphenyltetrazolium ions slowly, presumably because of the loss of co-factors. The extraction and estimation of the formazan was based on the methods of Kun and Abood (1949) and Fahmy and Walsh (1952). Fig. 4 shows that the inflexion point occurs earlier for the inhibited system (16 min. instead of 25) and hence that inhibition by mode II tetracycline can be accounted for by interference with hydrogen transfer at a step subsequent to that at which hydrogen is intercepted by triphenyltetrazolium ions. Similar results were obtained with 0·3 μM oxytetracycline.

The absence of inhibition by mode II in aerated cultures is also explicable. A branch of the transfer system must be capable of transferring hydrogen to oxygen as the final acceptor and only be operative when oxygen is available. If the point at which this branch begins preceeds the reaction inhibited then the inhibition cannot decrease the rate of removal of hydrogen from the initial source.

Mode I. The facts that, with both antibiotics, enriching the medium, including the addition of amino-acids, and aerating the cultures, do not

lessen mode I inhibition, suggests that this interference is with the utilisa-
tion of amino-acids for growth, and there are reports that the tetracyclines
derange the synthesis of protein (Eagle and Saz, 1955). Such an inter-
ference with the metabolism must result in other phenomena and the
experimental detection of such phenomena under mode I conditions would
be strong support for the suggestion.

During the logarithmic phase of growth of a culture, the metabolism
of an organism is in a steady state in which all the metabolic intermediates
have a constant concentration determined by the balancing of the com-
bined rates of all reactions producing a particular intermediate and
the combined rates of all reactions utilising that intermediate. Any
decrease in a rate of utilisation of an intermediate must result in a new
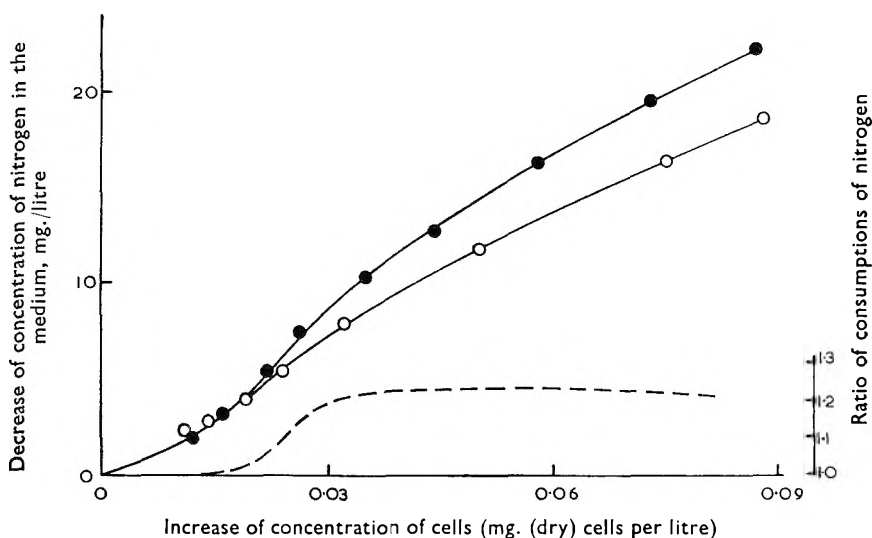equilibrium and a higher steady state concentration of the intermediate.

FIG. 5. Effect of tetracycline on the utilisation of nitrogen by *A. aerogenes*. Con-
centration of tetracycline (μM): ○, 0; ●, 0·10.

Thus a decrease in the rate of utilisation of amino-acids results in a higher
concentration of amino-acids. The change, however, must be small
since increase of concentration of amino-acids is likely to expedite other
utilisations and to depress their own rates of production. The latter in
turn causes an increase in concentration of the precursors of the amino-
acids. The net result is a period of adjustment during which the intake
of substances from the medium is maintained but the concentrations of
all intermediates rise and the output of some waste materials may also
increase. This period ends when the effect of the initial interference has
spread to the whole metabolism and the rate of intake of material from
the medium is decreased also and brought into balance with the combined
output of cells and waste materials. Measurements of concentrations of
any one intermediate or class of intermediate would have to be made with

extraordinary accuracy to detect the change, but during the period of adjustment the ratio of the amount of material taken in from the medium to the amount of cells produced is larger than it is in a normal culture. Since the change in this ratio during inhibition is a summation of all changes in concentration of intermediates and increases of rate of production of all waste products, experimental measurement of it should be relatively easy.

The weights of cells per litre and the remaining concentrations of ammonium nitrogen (micro-diffusion method, Conway, 1961, accuracy ± 1 mg. N/litre) were measured at intervals in two sets of six unaerated cultures at pH 7·00 containing initially 95 mg. N/litre; the cultures of one set contained also 0·1 μM tetracycline. The results for all like media were in close correspondence so that composite weight of cells per litre against time, and concentration of ammonium nitrogen against time, graphs could be constructed. From these graphs corresponding values cf weight of cells per litre and nitrogen consumed could be obtained. The results (Fig. 5) show that in fact, early in the growth phase in the presence of tetracycline a smaller proportion of the nitrogen taken in is utilised in the production of cells but that as the system stabilises the rate of intake of nitrogen decreases so that it more nearly matches its normal ratio to the rate of production of cells. In a similar experiment, aerated cultures containing oxytetracycline showed a similar deviation from the behaviour of the control cultures.

DISCUSSION

Attributing the inhibitory power of the two tetracyclines solely to their molecular forms TH_2 and $\emptyset H_2$, accounts quantitatively for the effects of varying the concentration of magnesium and the pH of the medium. Thus the relative effectiveness of the two tetracyclines car only be examined by comparing the degrees of inhibition due to the same concentrations of TH_2 and $\emptyset H_2$. This comparison is made in Fig. 6.

Mode I tetracycline and mode I oxytetracycline are indistinguishable and since neither are affected by enrichment of the medium, nor by aeration, but both produce phenomena expected from a derangement of protein synthesis, it is probable that both tetracycline molecules equally inhibit the same reaction in the sequence producing protein from aminoacids. This being so, this particular function is probably associated with a part of the molecule which is the same in both tetracycline and oxytetracycline.

The two mode II inhibitors are quantitatively different; that for oxytetracycline has a lower threshold concentration and the degree of inhibition rises less steeply with concentration. However, both are eliminated by aeration and by the same enrichment of the medium and both affect the reduction of triphenyltetrazolium ions in the same way. Thus it is probably that both tetracycline molecules inhibit the same hydrogentransfer reaction or the provision of a reducible substance. This being so, this particular function is probably associated with a part of the molecule which is affected by the difference of structure.

These considerations of molecular structure might well be expected if the primary step in inhibition is combination with enzymes; combination with one enzyme accounting for mode I and with another for mode II. The linearity or near linearity of portions of the Figs. 1, 2, 3 and 6 can be explained by the same hypothesis (Harris and Morrison, 1961).

Recent work with mice injected interperitoneally with tetracycline (Du Buy and Showacre, 1961), showed that the mitochondria of liver, spleen and brain cells became fluorescent and oxidative phosphorylation
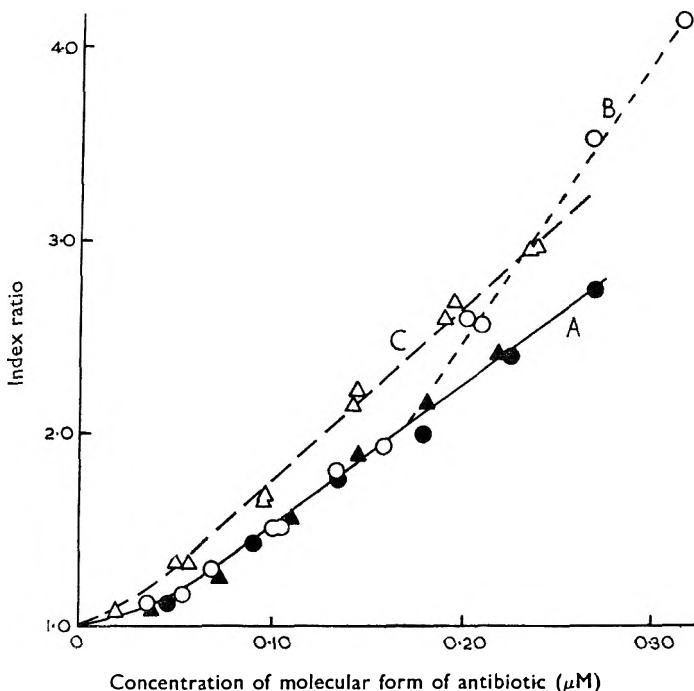

FIG. 6. Comparison of inhibitions of *A. aerogenes* by tetracycline and oxytetracycline. Cultures inhibited by tetracycline: ○, unaerated; ●, aerated. Cultures inhibited by oxytetracycline: △, unaerated; ▲, aerated. A, mode I, common to both antibiotics; B, mode II for tetracycline; C, mode II for oxytetracycline.

within them was decreased although oxygen uptakes were unaffected: the correspondence to the present mode II is obvious, and hydrogen-transferring enzymes or enzymes producing reducible substances might be expected to be present in the mitochondria.

In clinical cases any contributions of mode II to the cytotoxic action of these two tetracyclines can be only in tissues which do not have an adequate supply of oxygen, but mode I should contribute in all tissues that the antibiotics can reach. The power to combine with cations may be the cause of the absence of reports that these tetracyclines affect the faster growing cells of host animals such as blood cells which are affected by the inhibitor of protein synthesis, chloramphenicol: the tetracyclines may not be able

to reach the blood producing centres in the molecular inhibitory form because of the availability of calcium in the bone. Milch. Rall and Tobie (1957) report that interperitoneally injected tetracycline induces fluorescence in the bones of several species of laboratory animals, and we find that filtering an aqueous solution of tetracycline through powdered calcium ortho-phosphate severely decreases the inhibitory power.

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NEW APPARATUS

A SIMPLE MECHANICAL COMPUTER FOR RELATING THE CONTRACTILE RESPONSES OF TISSUES

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The principle of similar triangles has been applied to make a simple mechanical computer for relating the contractile responses of tissues.

ONE of the more tedious and time consuming aspects of many pharmacological experiments is the measurement of heights of contraction and the expression of these in terms of the maximal response of the tissue. This is particularly so when the prolific responses of smooth muscle are being measured. As the intention in most instances is to express the response as per cent of the maximal, so that experiments may be compared or collated, a simple method of doing this would be to mark the measure of the maximal response along the edge of a piece of card and to divide this measure as a per cent scale. Other responses of the same tissue could then be measured using this scale. This would be justified only where a large number of responses were being measured against the same maximum. The method has the obvious disadvantage that a new scale has to be prepared for each tissue. The cure is little better than the affliction.

An extension of this application is the use of the principle of similar triangles in the following way. In Fig. 1 we have a right-angled triangle ABC with a line DE drawn within the triangle, parallel to BC. If a line is drawn from A to meet BC at F transecting DE at G then

$$\frac{BF}{BC} = \frac{DG}{DE}$$

If BC is divided into a per cent scale then the relation per cent of BF to BC can be measured directly. In doing this we have also an indirect measure of the relation of DG to DE; thus if BF is 60 per cent of BC then DG must be 60 per cent of DE. This will be true for all positions of DE parallel to BC which fall within the triangle. The length of DE can be varied between zero and the length of BC. Similarly the angle BÂF can be varied between zero and BÂC. In this way, the transept of AF on BC (i.e., BF) will indicate the relation, per cent, of DG to DE for all values of DG and DE from zero to the length of BC.

If we now make DE the direct measure of the maximal response of our tissue then the position of AF can be altered so that the length DG corresponds with the length of any other response of the tissue and BF will give a measure of the relation of this response to the maximal.

Description of the Instrument

The instrument may be made of perspex sheet and consists of three parts, a flat triangular sheet inscribed on both sides with the triangle ABC (Fig. 1), a flat cursor which moves angularly about CÂB (and inscribed on both surfaces with a line, representing AF in Fig. 1) and a T-square, again with a line inscribed along both surfaces. When the T-square is in position the line on its surface represents DE in Fig. 1. The complete instrument is shown in Fig. 2. The cursor moves angularly below the lower surface of the triangle. The T-square is on the upper surface of the triangle with its cross-member lying in a channel along the base of the triangle. To make the instrument one unit the cross-member of the T-square is held proud to the side of this channel with a small leaf spring.
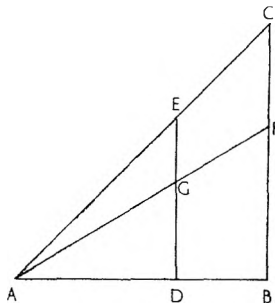

FIG. 1

The dimensions of the instrument will be dictated by the largest measurement to be made. In this department where maximal contractions up to 10 in. are recorded the side BC of the triangle is 12 in. The main body of the instrument is of $\frac{1}{4}$ in. perspex sheet. Two $\frac{1}{4}$ in. thick strips $1\frac{1}{4}$ in. wide along the straight edges raise the lower surface of the triangle above the tracing to be measured and allow the cursor to move freely between the tracing and the lower surface. The dimensions of the cursor are 3/16 in. × 1 in. × $19\frac{1}{2}$ in. with a line inscribed along the middle of each of the 1 in. wide surfaces.

The cursor is attached to the main body of the instrument by a countersunk bolt at the pivoting end and by a perspex overlap sufficient to permit free movement at the other.

The T-square is made of $\frac{1}{8}$ in. perspex and its main length and width are 13 in. and 1 in. respectively. The length of the cross-member of the T-square is about 4 in. and its width $\frac{7}{8}$ in. The channel in the main perspex sheet along which the cross-member slides is 1 in. wide and $\frac{1}{8}$ in. deep; the cross-member is held in this channel by a small steel leaf spring attached to the lower extremity of the T-square.

Method of Use

The instrument is placed in position over a tracing or other record so that the base of the triangle inscribed on the perspex (AB in Fig. 1)

coincides with the base-line of the record. The instrument is then moved along the base-line (maintaining the coincidence of the two base-lines) until the apex of the maximal response on the record meets the side of the triangle AC. The T-square is then brought into position so that the intercept of its inscribed line with AC lies immediately above the apex of the maximal response. The T-square will be held in this setting by its leaf spring and the setting will represent the maximal response of the tissue (corresponding to DE in Fig. 1). The entire instrument is then moved (still maintaining the coincidence of the base-lines) until the inscribed line of the T-square lies over the apex of one of the other
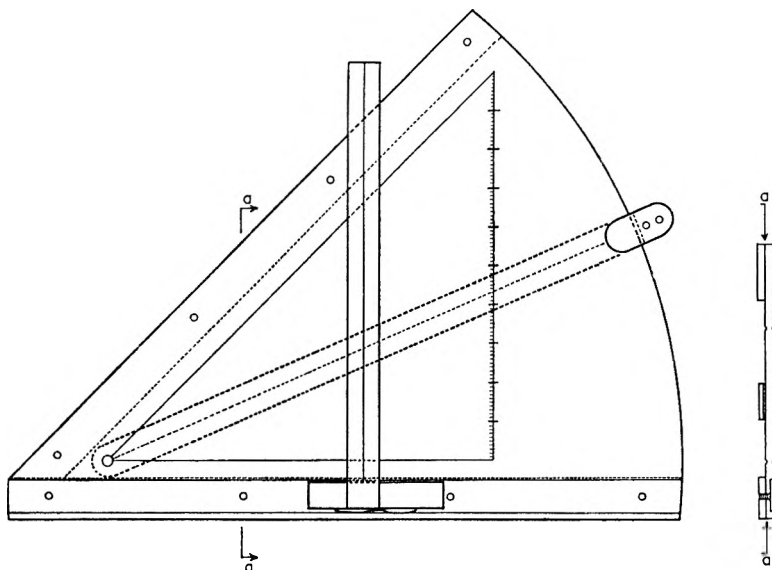

FIG. 2

responses of the tissue. The line on the cursor is now brought angularly into position over the apex of this response. The line on the cursor now corresponds with AF in Fig. 1 and the apex of the response is found to lie immediately under a point corresponding to the intersection G. Thus, should DE represent the maximal response of our tissue, DG now represents the response being computed. The per cent relation of DG to DE can then be read directly from the position of the intercept of the line of the cursor on the scale along BC. Parallax, arising from the thickness of the perspex between tracing and eye is avoided by duplicating the inscribed lines on the two relevant surfaces and aligning these lines.

The instrument was designed in the first instance for use with common pharmacological responses. It may also find application in other fields such as the comparison of measurements in photomicrographs and in computer record analysis.

LETTERS TO THE EDITOR

Drugs and Rat Pregnancy

Sir,—Whilst investigating the role of histamine in rat pregnancy, it became necessary to study the action of certain drugs in animals which had been mated. The results however may be of interest clinically as some of the drugs which are used in human pregnancy produced abortion in rats. No foetal abnormalities like those seen after thalidomide in humans and rabbits have so far been produced but this may only be because conditions for such malformations have not been achieved. Besides, experimental work on laboratory animals during the past 10 years has revealed that many chemical substances have the power when administered to the pregnant female of producing congenital malformations in the young (see Millen, 1962).

We tried to discover why the rat foetus forms much histamine during the last third of gestation and why the maternal urinary excretion of free histamine increases more than 10-fold during this period. The uterine and placental histaminase activity increases to about the same extent at this time yet the maternal uterus becomes relatively insensitive to histamine. Thus, the hypothesis was made that the function of histamine formed by the rat foetus is to help control the blood flow through the placenta (Kameswaran, Pennefather and West, 1962).

TABLE I

SUBSTANCES INFLUENCING RAT PREGNANCY (DOSES IN MG./KG. GIVEN DAILY SHOWN IN PARENTHESIS)

Drugs without effect	Substances with toxic actions
Histamine (100)	5-Hydroxytryptamine (10)
Lysergic acid diethylamide (1)	Mepyramine (50)
2-Bromolysergic acid diethylamide (4)	Promethazine (25)
1-Methyllysergic acid butanolamide (4)	Cyproheptadine (25)
Bretylium (15)	Aprobit (2)
Guanethidine (10)	Reserpine (0·5)
Aminoguanidine (20)	Chlorpromazine (30)
α-Methyl-dihydroxyphenylalanine (75)	Compound 48/80 (2)
Semicarbazide (20)	Polymyxin B (5)
	Compound L1935 (2)

In our first series of experiments, 24 pregnant rats were used. Daily subcutaneous doses of α-methyldihydroxyphenylalanine (75 mg./kg.) or of semicarbazide (20 mg./kg.) were given throughout pregnancy in an attempt to inhibit the histidine decarboxylase activity in the foetal liver. To inhibit uterine and placental histaminase in other rats, daily subcutaneous doses of aminoguanidine (20 mg./kg.) were given. However, neither treatment had any effect on the life-history of the litters although there was a slight reduction in the capacity of all the foetal livers to form histamine when foetuses from each group were examined about the 20th day of pregnancy.

In the second series 81 pregnant rats were used. Daily subcutaneous doses of histamine (100 mg./kg.) and daily intraperitoneal doses of specific anti-5-hydroxytryptamine drugs such as the three lysergic acid derivatives shown in Table I were found to be non-toxic to rat foetuses, and they too slightly reduced the enzyme activity in the foetal liver. In sharp contrast, both 5-hydroxytryptamine (10 mg./kg.) and the specific antihistamine drug mepyramine (50 mg./kg.) were toxic to foetuses although the histamine-forming

capacity of the foetal liver and the histaminase activity of the placenta were unchanged. Two other antihistamine drugs, cyproheptadine and promethazine, produced death of some of the foetuses when given intraperitoneally in high dosage (25 mg./kg.). The most toxic antihistamine drug tested was Aprobit, 2-hydroxyethyldimethyl-1-(10-phenothiazinylmethyl) ethylammoniumch loride; this quaternary drug fails to pass the placenta barrier and readily accumulates in the placenta where it may antagonise the vasodilator action of histamine which has been formed by the foetus. Aprobit is more than 10 times as toxic as promethazine in rat pregnancy, and at least 100 times more toxic to the foetus than to the mother. Although promethazine and Aprobit are toxic when injected into pregnant rats, they are both used orally in human pregnancy to alleviate nausea and vomiting; a warning note should therefore be sounded.
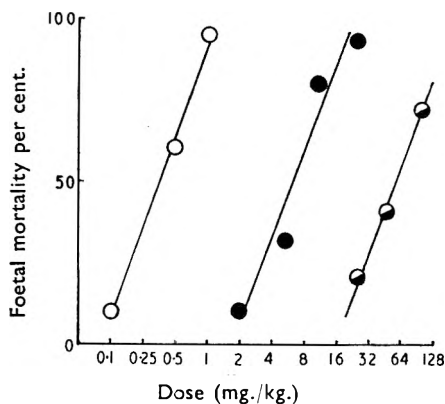

FIG. 1. The relation between log dose of reserpine (○), Aprobit (●) and promethazine (◐) and foetal mortality in rats.

In the third series 56 pregnant rats were used. The antihypertensive drugs, guanethidine (10 mg./kg.) and bretylium (15 mg./kg.), were given daily but no observable effects on the course of pregnancy or on the offspring were noted. In sharp contrast, daily doses of both reserpine (0·5 mg./kg.) and chlorpromazine (30 mg./kg.) were toxic, many foetuses being resorbed by the 20th day of gestation. These four drugs are now widely used in human pregnancy and care also seems necessary in their use. Various synthetic histamine liberators (for example, compounds 48/80 and L1935, and polymyxin B) produced abortion and death of some of the rat foetuses, but these drugs are not used routinely in man. Experiments upon animals such as these reported here may thus be valuable in determining which drugs are most likely to exhibit teratogenic activity in man.

Woollam (1962) has suggested that any drug which will kill the foetus will also deform it if given in a lower dose, and it may be that one of the most practicable methods of detecting teratogenicity is to relate the foetal toxicity to the toxic dose for the mother. Dr. G. F. Somers (in a personal communication) has suggested that it may be better to determine the foetal resorptions in relation to the dose administered to the mother (that is, not to the toxic dose for the mother). If this relationship is traced, say, for reserpine, Aprobit and promethazine (see Fig. 1), the lines are quite steep and there is only a narrow band of doses where teratogenicity is possible. The corresponding lines for

other drugs which have been shown to produce congenital malformations in the young may be flat so that a 10- to 20-fold increase in dose may produce only a moderate increase in toxicity.

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Anti-inflammatory Activity of Musk

SIR,—Musk is a dried secretion obtained from the prepucial follicles of *Moschus moschiferus* linn. (Fam. Cervidae.) (Chopra, 1958.) Practitioners of the indigenous system of medicine claim to obtain beneficial results with musk in arthritis, and we have now examined its anti-inflammatory properties.

Male albino rats weighing between 150–175 g. were subdivided into 5 groups. The hair of the back was removed with depilatory and the area washed and sterilised with ethanol. Three sealed musk pod samples of authentic musk obtained from the Institute of History of Medicine and Medical Research, India, were examined. Each was made into an emulsion in Tween 80, itself inactive, and injected subcutaneously in 10 rats for each dose of 1·0, 1·5 and 2·0 mg. for each of the 3 samples. Hydrocortisone suspension (Glaxo), 1 mg. in 0·1 ml., was injected subcutaneously to a group of 10 rats and another group of 10 rats kept as control. Granuloma pouches were made 24 hr. later in all the animals, under light ether anaesthesia, by injecting 25 ml. of air deep into loose subcutaneous tissue in the interscapular region using a No. 27 needle followed by the injection through the same needle of 1 ml. of one per cent croton oil solution in olive oil, into the resulting pneumodermal space. During the first 2 to 3 days, the pouches were essentially similar in all the groups. The changes began from the fourth day onwards. In the control group, the wall of the pouch began to thicken and haemorrhagic fluid started filling the cavity. In the musk- and hydrocortisone-treated animals, the wall of the pouch was very thin, transillumination revealing no haemorrhagic fluid and the gradual collapse of the pouch as the air was absorbed.

The experiment was terminated on the fourteenth day after the croton oil injection. The granuloma pouch was dissected, the amount of haemorrhagic fluid present was measured and the pouch wall weighed after careful washing. In the control animals, the pouch was filled by a large amount of haemorrhagic exudate and the pouch wall was extremely hard. In the musk- and hydro-cortisone-treated animals only a slight elevation of the skin remained, which indicated that a small amount of the air remained.

The results are given in Table I. The "*t*" test was done to determine the mean values and Snedecor's "F"-test to find out the significance of the difference between the three samples of musk. Since, at the same dose level, the samples gave statistically homogeneous results, they were pooled. The difference

in values between the control, musk- and hydrocortisone-treated animals are statistically highly significant (P < 0·001). After 1·0 and 1·5 mg., and 1·0 and 2·0 mg. respectively of musk, the difference in the amount of fluid was also significant (P < 0·01 and < 0·001), but betweeen 2·0 mg. musk and 1·0 mg. hydrocortisone the difference was not significant (P > 0·7), neither was the difference between the weights of the pouch wall significant, in these latter two instances.

TABLE I

EFFECT OF MUSK AND HYDROCORTISONE ON GRANULOMA POUCH

Drug	Dose (mg.)	No. of rats in each expt.	Fluid in the pouch wall (ml.)	Weight of the pouch wall (g.)
1. Control ..	—	10	10·4 ± 0·293*	4·8 ± 0·161*
2. Musk ..	1·0	30	1·8 ± 0·17	1·4 ± 0·094
(pooled	1·5	30	1·1 ± 0·17	0·8 ± 0·094
samples)	2·0	30	0·1 ± 0·17	0·3 ± 0·094
3. Hydrocortisone acetate ..	1·0	10	0·2 ± 0·293	0·4 ± 0·161

* All figures represent means of S.E. of the mean.

We believe these tests to show musk to be an anti-inflammatory agent like hydrocortisone acetate.

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Effectiveness of 5-Hydroxytryptamine in Ectopic Ventricular Tachycardia Resulting from Acute Myocardial Infarction in the Dog

SIR,—In an earlier publication, we showed that nialamide, a monoamine oxidase inhibitor reverted ectopic ventricular tachycardia induced by two-stage ligation of the anterior descending branch of left coronary artery in dogs to normal sinus rhythm (Kapila and Arora, 1962). Since monoamine oxidase inhibitors increase the concentration of various biological amines within the body tissues (Udenfriend, Weissbach, and Bogdanski, 1957), it was likely that at least one of these amines might be responsible for the observed salutary effect. Noradrenaline, however, when injected intravenously under the same experimental conditions as that of nialamide evoked ectopic ventricular beats (Maling, 1957), instead of reverting ventricular tachycardia to normal sinus rhythm. We therefore thought it worthwhile to study the effect of another monoamine (5-hydroxytryptamine (5-HT)) on the ectopic ventricular activity.

831

An aqueous solution of (5-HT) creatine sulphate was injected intravenously 18 hr. after two-stage ligation of the anterior descending branch of the left coronary artery of the dog (Harris, 1950), in doses of 10, 20, and 50 µg./kg. The electrical changes in the heart were observed through a cardioscope and the electrocardiogram recorded on a two channel Philips cardiopan 2.

Half an hour after the intravenous injection of 5-HT, there was a short phase of about 10–15 min., when there was a slight reduction in the ectopic ventricular rate after which it again returned to the previous level. During this period there was a slight increase in the total heart rate.

TABLE I

PEAK EFFECTS OF 5-HT CREATININE SULPHATE ON TOTAL HEART RATE AND ECTOPIC
VENTRICULAR RATE

Dose of 5-HT µg./kg.	Total heart rate/min.		Ectopic ventricular rate/min.		Toxicity
	Before drug	After drug	Before drug	After drug	
10	180	170	180	136	None
10	148	140	104	60	None
10	164	150	124	76	None
20	160	140	120	46	None
20	170	160	165	60	None
20	140	115	120	15	None
50	210	152	190	104	None
50	180	132	160	80	None
50	206	170	206	160	None
50	188	170	188	40	None
50	220	135	200	15	None
50	176	150	150	32	None

Maximum reduction in the ectopic ventricular rate and total heart rate occurred 24 hr. after the drug administration. There was a decrease in the total heart rate and ectopic ventricular rate (Table I). A dose response relationship was not noted, probably due to the higher doses employed in dogs having initial higher total heart rate and ectopic ventricular rate. The peak effect lasted for more than 8 hr. 40 hr. after the drug administration ectopic ventricular beats were present in large number.

The present studies reveal that 5-HT when injected intravenously in dogs having ventricular tachycardia produced its effect in two phases. Firstly, an immediate effect of short duration with some reduction in ectopic ventricular rate and a slight increase in total heart rate. Secondly, the peak effects were noted 20 hr. after 5-HT administration. There was a reduction in the total heart rate as well as in ectopic ventricular rate and the effect lasted for a number of hours.

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BOOK REVIEWS

THE DIAGNOSIS AND TREATMENT OF ACUTE POISONING. By
J. D. P. Graham. Pp. viii + 438 (including Index). Oxford University Press,
London, 1962. 45s.

It is difficult to write a good book on the management of poisoning. From
a theoretical aspect, the distinction between the therapeutic and the toxic actions
of a drug is often only quantitative, and there is little value in considering one
without the other. From a practical point of view, poisoning usually requires
urgent treatment if it requires any treatment, and a discursive handbook is worse
than useless. What is wanted is a reliable and brief guide to diagnosis and
treatment, from which the necessary facts can be elicited accurately in a minimum
time. It is possible that casualty officers and others may welcome a book
which comes between the two in scope, and is at once readable and concerned
only with practical problems. For these, this book is written, in characteristic
style and from Dr. Graham's considerable personal experience. It has the
advantages and disadvantages of accounts which depend on personal experience
rather than on statistically balanced evidence. It is to be regretted that the
appendix which lists numerous proprietary preparations of drugs consistently
omits to specify the identity of more than one ingredient in a remedy. It seems
undesirable, for example, to embark on treatment of poisoning with "Drinamyl"
without knowing that it contains dexamphetamine, or with "Barbasprin" or
"Dexytal" without awareness that they contain barbiturates. It is also incorrect
to refer to amphetamine sulphate, cocaine, and codeine sulphate as proprietary
names, but this may not matter in the urgency of treating a poisoned patient.

MILES WEATHERALL.

DRUGS AFFECTING LIPID METABOLISM. Proceedings of the Symposium
on Drugs Affecting Lipid Metabolism, Milan, 1960. Pp. viii + 604 (including
Index). Elsevier Publishing Company Ltd., London, 1961. 126s.

Congresses, symposia, conferences, and other forms of discussion are vital
to co-ordinating and correlating research problems, and reports on their pro-
ceedings are of interest to all non-participants who are directly concerned with
the subject. However, it is essential that the information reported at these
meetings is made available as soon as possible otherwise its value is considerably
lessened. Perhaps the rather expensive bindings and high standards of printing
often encountered should be sacrificed for speed in getting the information out
to research workers.

Drugs Affecting Lipid Metabolism, edited by S. Garattini and R. Paoletti,
comprises more than 80 papers and short communications read at the Symposium
in Milan in 1960. Whilst there are contributions in Italian, French and German,
most are in English and all are summarised in English. Intensive research
work has been conducted in recent years into lipid metabolism and a significant
contribution has come from pharmacologists: this book surveys the most recent
ideas and advances in this field. The book is divided into four main sections.
The first deals largely with the biosynthesis of fatty acids and their incorporation
into lipid molecules, the biosynthesis and catabolism of cholesterol and the
absorption and excretion of these substances. It is unfortunate that the recent

brilliant work of Kennedy on the biosynthesis of glycerides and phospholipids was not discussed. The volume does not include points raised during discussions on each paper: in my experience this part of a symposium is usually most illuminating and instructive.

The second section deals with the experimental methods for the evaluation of drugs affecting the metabolism of the substances mentioned earlier. Physical methods, including vapour-phase chromatography and column chromatography, are described for the separation of steroids and fatty acids, but investigations with labelled substances are surprisingly few. The mechanism of the inhibitory effects of MER-29 on cholesterol metabolism and the bearing of these findings on the place of desmosterol in the biosynthesis of cholesterol is reported. Another paper on the comparative evaluation of hypocholesteremising drugs, including MER-29, thyroxine, nicotinic acid, diphenesenic acid, benzmalacene, and their related compounds indicates that each group has quite a different pattern of activity, which suggests there are various modes of action.

Section three is devoted to the important current problem of drugs affecting the lipid metabolism in relation to experimental atherosclerosis. The effects of thyroxine and compounds including steroids and other natural products on the disease and also the lowering of cholesterol level by the use of isoprenoids and corn oil are reported. The possibility that genetic factors are involved in the level of circulating cholesterol by exerting their influence on controlling cholesterol absorption is suggested by two Canadian workers.

The last section, devoted to the therapeutic value of drugs, is probably the most important, and should have revealed interesting observations on discussion. Most papers read in this section are concerned with clinical trials reporting the lowering effects and other alterations of lipid metabolism; evidence from *in vitro* experiments using labelled substances is also presented.

In conclusion, the symposium showed that there are now very powerful methods of achieving a lowering of serum cholesterol. Elucidation of the mechanism of inhibition of biosynthesis at various stages is illustrated by the use of isotopes. Other ways of lowering serum cholesterol by, for example, increased oxidation and excretion are outlined. A controlled diet could also achieve some effect, but Professor Sinclair raises the question whether these are desired effects to be produced in isolation from structural and other functional roles. His contribution is most valuable.

This volume is beautifully printed and bound. It is a book for the specialist, and this limitation keeps its price out of reach of many interested workers.

N. ROBINSON.

THE RUSSIAN DRUG INDEX. National Library of Medicine*. Washington 25, D.C. 1961. U.S. Public Health Service Publication No. 814. Pp. 103 (including Index).

The book is divided into two parts: the first gives information on a large number of drugs encountered in Russian medical and pharmaceutical literature; the second, forming the index proper, is an alphabetical list of names, synonyms and cross-references.

In the main subject section, the drugs are classified under some fifty functional headings. The entry under each drug begins with the anglicised form of the "Latin" name; this is followed by the transliterated Russian name, the chemical formula or composition, the structural formula, properties, a Russian reference and an American reference. For the Russian references, the authors have

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drawn heavily on Mashkovskii's *Legarstvennye sredstva*, the most useful source of information for readers who know some Russian, but there are also numerous references to the journal literature. The system used for transliterating Russian names is similar to that used by *Chemical Abstracts*, which will be appreciated by research chemists.

The book is an essential reference for all workers who have to deal with Russian medical or pharmaceutical literature, and for pharmacists who may have to decipher Russian prescriptions.

E. HAYES.

PRINCIPLES AND APPLICATIONS OF PAPER ELECTROPHORESIS. By Ch. Wunderly. Pp. xii + 253 (including Index). Elsevier Publishing Company Ltd., London, 1961. 26s.

Paper electrophoresis was in its infancy before 1950 but since then it has expanded and improved rapidly as an analytical tool for chemists and workers in the biological sciences.

The method of free electrophoresis had been applied to problems in clinical medicine for some time before the advent of paper electrophoresis, and it was to be expected that the development of the paper method was associated with clinical investigations. The simplicity of the method and its suitability for the micro scale makes it particularly applicable to the study of the composition of biological fluids during the course of illness. More recently the technique has been applied to pharmacology, experimental pathology, enzymology and botany. In his book, Dr Wunderly has discussed problems in all of these fields and, in addition, he has mentioned the little explored use of paper electrophoresis in inorganic chemistry.

In this compact volume, Dr. Wunderly has gathered together information drawn from many publications, which he has carefully selected for their reliability. A short introduction leads into a chapter on theoretical aspects emphasising the fundamental physico-chemical concepts of migration and the rate-determining factors. These are treated in non-mathematical terms and stress the practical aspect.

In the succeeding chapter the author reviews the methods which use low and high voltages and different arrangements of support for the stationary phase, and also the kinds of paper to select for different problems. More recent developments such as column electrochromatography, other modifications to vertical migration, and "continuous deviation" for the separation of larger quantities of materials into their components are adequately covered. This last technique is too expensive for most laboratories. Many of the kinds of apparatus described are of foreign manufacture: the names of British manufacturers should have been quoted where they exist.

Chapter 4 describes in detail the practice of electrophoresis in the laboratory. Many examples are given, together with advantages and some of the difficulties that may arise. An invaluable ten pages are devoted to the statistical interpretation of results. The author of "Histochemistry," referred to on page 50, should read A. G. Everson Pearse.

More than half the book is devoted to the last chapter describing the results achieved by electrophoresis. These include the isolation and examination of highly complex molecules such as vitamins, nucleic acids, enzymes, lipoproteins, antibodies and also viruses and toxins. 'Labelled' substances are also discussed. Very often sufficient experimental detail is given to enable the reader to conduct his own experiments without recourse to original work. This is not the primary purpose of the author, the book is too compact to achieve this, but the information, whether in detail or in the bibliography, is there.

The author deserves praise for the amount of information he has put into this small volume, at the same time keeping it lucid and well illustrated with excellent photographs and diagrams. An extensive list of references (1721) will make this book a standard reference on paper electrophoresis. It is excellent value at 26s.   N. ROBINSON.

PRACTICAL PHARMACEUTICAL CHEMISTRY. Quantitative Analysis By A. H. Beckett and J. B. Stenlake. Pp. viii + 378 (including Index). University of London, The Athlone Press, London, 1962. 63s.

From earliest times the notion that education is more concerned with values than with facts has persisted. Homer in his "Iliad" uses the descriptions of the armour of the warriors to inculcate ideas of excellence in workmanship.

This is no less true in analytical chemistry which must be taught so as to produce in the student attitudes of precision and care in experimental work. The educational value of this then extends far beyond the boundaries of analytical chemistry.

Beckett and Stenlake's book on quantitative analysis will form an excellent basis for training along these lines. It has the outstanding merit of giving meticulous attention to the fine details of technique and procedure which make for accurate results. The attention to detail is such that the reviewer begins to search for omissions and finds that a reader is not warned that in gravimetric analysis a dried precipitate can rapidly increase in weight on the balance pan. The authors presume a knowledge of the analytical balance and there is therefore no mention of sensitivity, of the calibration of weights, or of modern aperiodic balances.

Although descriptions of analytical procedures are not expected to have the vividness of a novel they are sometimes in this book unnecessarily wordy. "To effect complete solution of the oil" means no more than "to dissolve the oil".

The part of the book which deals with physical instruments gives basic theory followed by details of experiments designed to illustrate the theory and the application of instruments in quantitative analysis. Again the standard is high and a reviewer can point out only small sections of the work which might be enlarged or clarified. With the exception of fluorescence spectrophotometry and possibly quantitative infra-red spectrophotometry all the important instrumental methods of analysis are well covered.

Other topics in this book which might not be inferred from the above account are chemical purity and limit tests, titrations in non-aqueous media, complexometric analysis and ion exchange.

It is clearly outside the scope of this work to refer to the original literature of the various analytical procedures and this has not been attempted. A future edition might, with advantage, include a chapter giving an account of the literature of analytical chemistry and an assay having an interesting theoretical background which could be critically discussed in the light of published literature. The iodometric assay of copper sulphate would serve the purpose well.

The reviewer would like to see formulae for calculating iodine and saponification values deleted. These are memorised by weak students and often applied with disastrous results. There is no reason why these values should not be calculated from first principles.

The authors are to be congratulated on their excellent work in compiling and classifying the large variety of analytical procedures, and the proof readers on the high standard they have achieved.

The book is strongly recommended for degree and diploma students in pharmacy and suggested as a reference work for analysts.   V. ASKAM.