JOURNAL OF PHARMACY AND PHARMACOLOGY

VOLUME XV No. 2 FEBRUARY 1963

Published by Direction of the Council of

THE PHARMACEUTICAL SOCIETY OF GT. BRITAIN

17 BLOOMSBURY SQUARE, LONDON, W.C.1

NOTICE TO CONTRIBUTORS

GENERAL. Original research papers or review articles are accepted on the understanding that the material has not been previously published. The title page should bear the name of the contributor and the name and address of the laboratory where the work was done.

PREPARATION OF TEXT. Authors should consult a current issue of the Journal and conform to the typographical conventions, use of headings, lay-out of tables, and citation of references. Texts must be typewritten in double spacing on quarto or foolscap sheets with a one-and-a-half inch margin. The top copy and one carbon copy should be sent. The following style should be used: Summary, introduction, experimental methods, results, discussion, acknowledgements, references. The summary should be brief but self-explanatory, giving results and conclusions described in the paper, and should be in the form of an abstract suitable for use as such by abstracting journals.

REFERENCES—CHANGE IN STYLE

References should be arranged according to the HARVARD system. In the text the surname of the author(s) and the date of publication are given thus:

Beleslin and Varagić (1958) described . . . or

. . . has been described (Beleslin and Varagić, 1958).

At the end of the paper the references are arranged in alphabetical order of the first author of each paper, and should include each author's name and initials, year of publication in parenthesis, title of publication (underlined) abbreviated as in the *World List of Scientific Periodicals*, the volume number, and the first and last page number of the paper, e.g.

Beleslin, D. and Varagić, V. (1958). *Brit. J. Pharmacol.*, **13**, 321-325.

References to books should follow the pattern: name of author(s), year of publication, title (underlined), editor's name or number of edition, place of publication, and publisher, e.g.

Goodman, L. S. and Gilman, A. (1955). *The Pharmacological Basis of Therapeutics*, 2nd ed., p. 213, New York: Macmillan.

TABLES (for each copy of the text) should be typed on separate sheets, their headings should describe their content and they should be understandable without reference to the text.

ILLUSTRATIONS. Illustrations, which need not be duplicated, are usually limited to 6 in number. They will normally appear in print between 2 in. and 4 in. in width (base line). Line illustrations such as graphs or apparatus should be clearly and boldly drawn at least twice the size of the reproduction required, in black waterproof ink on white paper, Bristol Board, faintly blue-lined paper or blue tracing cloth, at a thickness which will produce a suitable line when the scale is reduced. Kymograph records and photographs should be selected to allow for similar reductions. Lettering and numbering should be reduced to the minimum and inserted lightly and clearly in pencil. Curves based on experimental data should carry clear and bold indications of the experimentally determined points, which should be marked by using, preferably, circles, crosses, triangle or squares. Legends, descriptions or captions for all figures should be typed on separate sheets of paper and fastened to the typescript of the paper or attached to the individual figures. The author's name, the title of the paper and the number of the figure should be written on the back of each illustration. The approximate position of each illustration should be marked in the text. All illustrations should be understandable without reference to the text.

SYMBOLS AND ABBREVIATIONS. Authors should refer to the current number of the Journal for information. The attention of authors is drawn particularly to the following symbols: m = (milli) = 10^{-3} and μ = (micro) = 10^{-6}; ml. (millilitres) should always be used and not c.c.; μg. (micrograms) and not γ; per cent and not %.

NOMENCLATURE OF PLANTS, ANIMALS AND MICRO-ORGANISMS. Binominal Latin names, the generic names only with an initial capital letter, should be used in accordance with international usage. They should be underlined in the text. The first mention should be in full and the subsequent references abbreviated, but ambiguities should be avoided.

REPRINTS. 50 reprints are supplied free of charge to the author. A further 10 reprints are supplied free to each co-author. Additional reprints may be purchased.

COPYRIGHT. Contributions are accepted for publication on the condition that an exclusive licence to publish in any periodical form is granted by the contributor, in so far as he can grant the same, to the *Journal of Pharmacy and Pharmacology*.

The proper abbreviation for this Journal is *J. Pharm. Pharmacol.*

JOURNAL OF PHARMACY AND PHARMACOLOGY

Editor: George Brownlee, D.Sc., Ph.D., F.P.S.
Assistant Editor: J. R. Fowler, B.Pharm., F.P.S.
Annual Subscription **£5 0s. 0d.** Single Copies **10s.**
17 BLOOMSBURY SQUARE, LONDON, W.C.1
Cables: Pharmakon, London. W.C.1. *Telephone:* HOLborn 8967

Vol. XV No. 2 **February, 1963**

CONTENTS

REVIEW ARTICLE

THE ESTIMATION OF PENICILLINS AND PENICILLIN DESTRUCTION

By J. M. T. Hamilton-Miller, B.A., J. T. Smith, Ph.D. and R. Knox, M.D., F.R.C.P.

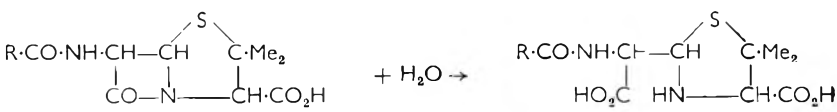
*Department of Bacteriology, Guy's Hospital Medical School, London, S.E.*1

Research into the biological and chemical aspects of penicillin and its production has been actively pursued for almost 35 years; the announcement of the production of the penicillin "nucleus", 6-aminopenicillanic acid, on a commercially attractive scale (Batchelor, Doyle, Nayler and Rolinson, 1959) has given a tremendous stimulus to these studies in the past few years. With the increased range of penicillins now already available for clinical use, and the possibility of further compounds with new specificities in the future, there is a new need for a review of methods for estimating penicillins and penicillin-destroying enzymes. This review covers chemical, physical and microbiological methods and attempts to indicate which of these is most suitable for the varying needs of present day research in this field.

Penicillin-destroying enzymes

Penicillinase

An account of the distribution of these enzymes is given by Abraham (1951). The enzymes act by rupturing the β-lactam ring of penicillins, and perhaps should be more precisely called β-lactamases (Pollock, 1961), as shown in equation 1.



Penicillin — Penicilloic Acid

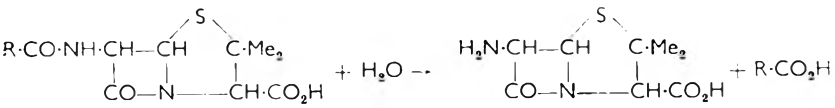
Equation 1

The antibacterial activity of the penicillin is for all practical purposes destroyed by this cleavage, which can also be brought about by alkali treatment. The nomenclature of the penicilloic acids, unlike that of the penicillins (Sheehan, Henery-Logan and Johnson, 1953) has not been strictly defined. "Penicilloic acid" is, strictly speaking, a general name and should be qualified, for example, "benzylpenicilloic acid", or "penicilloic acid G" derived from benzylpenicillin: however, the term has unfortunately been much used to refer specifically to the hydrolysis product of penicillin G. The use of trivial names, such as "methicilloic acid" from methicillin and "penicic acid" (Huang and others, 1960; Murao, 1955) from 6-aminopenicillanic acid, is hardly a satisfactory compromise. It would appear that the best solution to this problem of nomenclature of the penicilloic acids is to qualify the name of each individual compound by the full side-chain, for example, phenylacetamidopenicilloic acid from penicillin G, phenoxypropionamidopenicilloic

acid from phenethicillin, and aminopenicilloic acid from 6-aminopenicillanic acid.

Amidase

The properties and distribution of these enzymes, now of great commercial importance, are described by Batchelor and others (1959), Rolinson and others (1960), Claridge, Gourevitch and Lein (1960) and English, MacBride and Huang (1960). The latter authors call the enzymes "acylases". They act by removing the side-chain attached in the 6-amino-position of the penicillin nucleus.



Penicillin — 6-Aminopenicillanic Acid

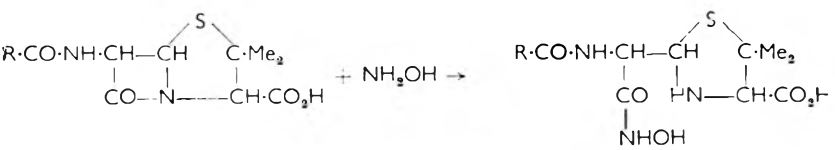
Equation 2

Unlike penicillinases, amidases catalyse both the forward and the backward reaction, and also do not completely destroy antibacterial potency, since the product of the reaction, 6-aminopenicillanic acid, also possesses some limited biological activity (Rolinson and Stevens, 1961).

Quantitative assays

Chemical

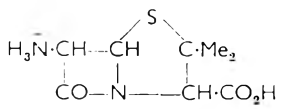
Hydroxylamine (Staab, Ragan and Binkley, 1946). This original method has been modified by Boxer and Everett (1949) and Ford (1947), amongst others. Hydroxylamine reacts with penicillins at pH 7·0 as shown by equation 3.



Equation 3

This reaction is complete within about 10 min. at room temperature and at pH values between 6 and 8. The product is a hydroxamic acid, stable for 2 hr., which forms a coloured internal salt with ferric ions, of unknown structure. This chromogen is stable for about 5 min., but can be further stabilised (Henstock, 1949) by extraction into n-butanol; its absorption is proportional to the concentration of penicillin, between 10–2,000 μg./ml., and can be measured colorimetrically at 500 mμ (Boxer and Everett, 1949; Batchelor, Chain, Hardy, Mansford and Rolinson, 1961). A spectrophotometer can be used, but this is inconvenient because of the short life of the chromogen, and because the method involves the mixing of alcoholic and aqueous solutions, and many gas bubbles are hence formed. The individual penicillins produce, mole for mole, differing colour intensities, and there does not seem to be any

simple relationship between chemical structure and chromogenicity of the hydroxamate-ferric complex. Hydroxylamine will react only with the molecular species having an intact β-lactam ring: hence during penicillinase assays, the substrate which remains rather than the product formed is being measured. Penicillin amidase activity cannot be measured directly by this assay, as the 6-aminopenicillanic acid produced by the hydrolysis still has an intact β-lactam ring. A method for the estimation of this enzyme is described by Batchelor, Chain and Rolinson (1961). At pH 2, 6-aminopenicillanic acid has a net positive charge,



while other penicillins are unionised, and exist as free acids which, being hydrophobic, are extractable into a suitable non-polar solvent, as for example, n-butyl acetate. The hydrophilic 6-aminopenicillanic acid remains in the aqueous phase, where it can be assayed by the hydroxylamine method.

Hydroxylamine does not react only with the penicillins. Hydroxamates are also formed with compounds containing a carbonyl group such as esters, when a purple chromogen is formed with the Fe^{3+}. For example, Lipmann and Tuttle (1945) describe the use of this assay for acyl phosphate determination at acid pH values. Despite this lack of specificity, however, the method is valuable for obtaining absolute rates of breakdown of penicillins, specificity patterns of different enzymes, or Michaelis constants (see Batchelor, Cameron-Wood, Chain and Rolinson, 1961; Knox and Smith, 1962, for examples). The chief disadvantage is the lack of sensitivity, as the lower limit for most penicillins is 20 μg./ml. even under the best conditions. This method has been adapted for automatic operation by Niedermayer, Russo-Alesi, Lendzian and Kelly (1960), for estimating up to 10,000 units/ml. (6 mg./ml.).

Iodometric (Alicino, 1946; Tucker, 1954; Perret, 1954). Penicilloic acids take up iodine; the stoichiometry of the reaction has not been worked out—it is known only that 8–9 atoms of iodine are taken up by each molecule of benzylpenicilloic acid. Penicillins which possess an unsaturated aliphatic side chain, such as penicillin O (allylmercaptomethyl) (Grove and Randall, 1955), and penicillin F (Δ^2-pentenyl) will reduce iodine while the β-lactam ring is intact; *p*-hydroxybenzylpenicillin also behaves in this way (Sneath and Collins, 1961). Iodine is absorbed by many other organic compounds, especially those that are unsaturated, and careful blank estimations must therefore be made. But as most unchanged penicillins do not reduce iodine, the amount of it taken up depends on the amount of penicilloic acid formed. Comparative assays using the hydroxylamine and iodometric methods give closely similar rates of hydrolysis by penicillinases (Boxer and Everett, 1949). The iodometric method cannot be used to follow hydrolysis by amidase, as 6-aminopenicillanic acid does not reduce iodine.

Perret's method estimates iodine uptake by sampling the reaction mixture into excess iodine, and back-titrating with standard thiosulphate. Citri (1958) uses a simplified version, in which the time taken for a standard amount of blue starch-iodine complex to be totally decolorized is used as a measure of reaction velocity. Weiss (1959) has reviewed the factors affecting the accuracy and sensitivity of the macro-iodometric assay.

Newer micro-iodometric assays, depending upon absorption by the I_3^- complex at 360 mμ (Goodall and Davies, 1961) and at 420 mμ (Ferrari, Russo-Alesi and Kelly, 1959), and on the absorption of the starch-iodine complex at 600 mμ, have been developed. The automatic method described by Goodall and Davies (1961), has been modified by Beecham Research Laboratories (Batchelor, F.R., personal communication). Novick (1962a,b) also used the starch-iodine complex as the chromogen, measuring absorption at 620 mμ with a spectrophotometer. He found that his micro-method gave 40 per cent lower activities for penicillinase than those obtained by the macro-method. The methods using the starch-iodine complex are very sensitive; about 1 μg./ml. of penicillin can be accurately estimated. Novick (1962a) has used his assay to determine the K_m of penicillin G for staphylococcal penicillinase, and it can obviously be extended to measure other K_m values for penicillins with especially high affinities for penicillinase.

Other methods. Penicilloic acids, unlike nearly all other organic compounds, will reduce arsenomolybdate at room temperature, in the presence of traces of mercuric chloride, to a chromogen "molybdenum blue" which is measurable colorimetrically (Pan, 1954). A semi-automatic modification has been described (Green and Monk, 1959).

Hiscox (1949) developed a method whereby the amount of ferricyanide reduced to ferrocyanide by penicillins is measured by back-titration with ceric sulphate. The assay can be used only for crystalline penicillins.

Scudi (1946) found that penicillins react with *N*-(1-naphthyl-4-azobenzene)ethylenediamine to form a red chromogen, which is measured at 540 mμ. The same author has also developed a very sensitive fluorimetric procedure (Scudi and Jellinek, 1946).

Acidimetric

When penicillins are hydrolysed by penicillinase a new carboxyl and a secondary amino-group are generated (equation 1). The latter, being weak, has little or no effect on the net production of hydrogen ions and hence the pH falls in proportion to the amount of hydrolysis. Benedict, Schmidt and Coghill (1945) found, by electrometric titration, that the pK of the newly-formed carboxyl group, in the case of benzylpenicillin, was 4·7 (compare the pK of the original carboxyl group, 2·16). Batchelor, Chain, Hardy, Mansford and Rolinson (1961) similarly found the pK of the penicilloic acid of 6-aminopenicillanic acid to be 3·7.

When the side chain is removed from a penicillin by hydrolysis with an amidase (equation 2), a new carboxyl and a primary amino-group are formed. Here the amino-group is sufficiently strong to absorb hydrogen

ions and hence the pH falls progressively more slowly as the reaction proceeds. Initial velocities can be taken by the usual method of drawing tangents, and hence reaction velocities, and Michaelis constants, could be calculated.

Comparison of rates of hydrolysis of different penicillins by penicillinase cannot be directly undertaken by these methods as different penicilloic acids are likely to have different pK values so that the fall in pH per molecule hydrolysed will differ for each penicillin.

The rate of production of hydrogen ions, and hence the rate of hydrolysis of penicillin, can be determined in the following ways.

Manometric assay (Foster, 1945). This method was standardised by Henry and Housewright (1947) and was modified further by Pollock (1952); it depends on the fact that the volume of CO_2 liberated from a bicarbonate buffer as penicillin is hydrolysed to penicilloic acid is proportional to the rate of hydrolysis. Manometric methods are time-consuming, especially where gassing is necessary; but many workers (Pollock, 1952; Pollock and Torriani, 1953; Steinman, 1961a, b; Crompton and others, 1962; Leitner and Cohen, 1962; and others) still use this assay.

Retention of either CO_2 or hydrogen ions will result in diminished gas evolution and thus cause misleading results. The results of Steinman (1961a) show that the hydrolysis of 6-aminopenicillanic acid by penicillinase cannot be estimated manometrically. Hydrolysis of 6-aminopenicillanic acid results in the formation of not only a carboxyl group but also of an α-amino group, and hence there is no net production of hydrogen ions. Furthermore Batchelor, Gazzard and Nayler (1961) showed that CO_2 reacts with 6-aminopenicillanic acid forming first a carbamate then a penillic acid.

The method is mainly used for the assay of penicillinases, although originally described also for penicillin assay. It appears that, at present, this assay method is in the process of being superseded by other methods.

Alkaline titrimetric methods. Patterson and Emery (1948) described a method for the estimation of benzylpenicillin in which the amount of standard alkali required to split the β-lactam ring was measured by back-titration with acid. Murtaugh and Levy (1945) used a pH stat for the same purpose, and Jeffrey, Abraham and Newton (1961) used this principle to follow the alkaline hydrolysis of cephalosporin C. The pH of the reaction mixture is kept constant by the automatic addition of sodium hydroxide solution, the rate of addition being proportional to the rate of hydrolysis. The latter method has also been used by Crompton and others (1962) to follow the course of alkaline hydrolysis of penicillin G, methicillin and deacetyl cephalosporin C.

Indicator method. The rate of change in pH is followed, in this method, by means of an indicator. The absorption of the alkaline form of the indicator at a certain wavelength will decrease as more penicilloic acid is formed. The indicator of choice is that whose pK coincides roughly with the pH at which the reaction is carried out (for example, phenol red pK = 7·8 at pH 7·6; bromothymol blue pK = 7·0 at pH 7·0; bromcresol

purple pK = 6·2 at pH 6·5, etc.). The method of Nirenberg (cited by Saz, Lowery and Jackson, 1961) measures the decrease in the red form of phenol red by determining the absorption at 558 mμ with a spectrophotometer. Work in this laboratory with bromothymol blue and bromcresol purple has shown the maximum absorption of the alkaline forms of these indicators to be at 645 mμ and 615 mμ respectively: the sensitivity of the method is shown by the fact that, in the presence of M/240 phosphate buffer and 0·0013 per cent (w/v) aqueous phenol red, a change in absorption of 0·001 unit (the smallest change that can be accurately detected) represents a change in pH of 0·0012 units. The absorption of the indicators changes in an almost linear fashion with pH over a reasonable range (for example between pH values of 7·8 and 7·1 for phenol red), and when following hydrolytic reactions, straight line progress plots are obtained. The method can also be used with a colorimeter to follow the reaction: this increases the flexibility of the assay.

Michaelis constants can be determined conveniently with this assay, but probably its greatest use is in detecting changes in reaction velocity (see Saz and others, 1961, for action of activators on staphylococcal penicillinase), particularly in relation to inhibition studies, which, up to now, have been studied mainly by the manometric method (Abraham and Newton, 1956). By virtue of its far greater convenience and increased ease of operation, it is possible that the indicator method may take the place of manometric methods in this respect.

Miscellaneous

Microbiological assay. The "Oxford cup method" was first described by Abraham and others (1941) and further developed by Heatley (1944). It has been modified by Schmidt and Moyer (1944), McKee, Rake and Menzel (1944), and Foster and Woodruff (1943b, 1944). The latter (1943a) also gave a carefully reasoned critique of various microbiological methods and finally recommend the Oxford cup method; in addition, Heatley (1948), Lees and Tootill (1955) and Kavanagh (1960) have reviewed this topic. The theory of diffusion of substances through agar has been worked out by Cooper and his associates (Cooper, 1955: Cooper and Linton, 1952; Cooper and Woodman, 1946).

The method is valuable for the determination of the antibacterial activity of clinical specimens and particularly useful for the investigation of the inactivation of penicillins by proteins, and it is more sensitive than any chemical method to date. Microbiological assay methods, however, are not particularly accurate, require many controls and are time-consuming. In addition, enzymic destruction of penicillins cannot be studied since there is no method available to stop the reaction which would not also interfere with the biological assay.

The choice of test organism is important when dealing with penicillins active against organisms which are relatively resistant to penicillin G. *Sarcina lutea* is widely used because of its high sensitivity to penicillin G and *B. subtilis* and *Staph. aureus* are also often used. But with ampicillin, which possesses activity against Gram-negative organisms, perhaps it

would be better to use a Gram-negative organism, and with penicillinase-resistant penicillins a penicillinase-producing organism may be a better choice as indicator strain. This could be vital when a penicillin is altered in the body (see Rollo, Somers and Burley, 1962) to become a compound which may be active against a badly chosen indicator organism but not active against the organism for which the penicillin was designed.

Spectrophotometric methods. Various direct spectrophotometric methods were described in the early days of large-scale penicillin production, mainly for estimating the amount of penicillin in fermentation liquors. Methods utilising absorption in the ultra-violet range have been reviewed by Colon, Herpich, Neuss and Frediani (1949) and Twigg (1949). The latter also discusses infra-red methods.

Polarimetric method. Abraham and Newton (1956) followed the alkaline hydrolysis of penicillin G by the change in optical rotation as sodium penicillin G was converted into sodium D (+)-α-benzylacetamidopenicilloate. The method is complicated by the fact that mutarotation of the product occurs.

Qualitative assays

Filter membrane method (Knox and Smith, 1961). This is a simple qualitative test for the production of acid by colonies when incubated for a short time with penicillin G and is a modification of the pour-plate method of Manson, Pollock and Tridgell (1954). Organisms grown on membranes are transferred to a solution of penicillin G containing Andrade's indicator: acid-producing colonies take up a red colour; controls can be tested without penicillin. Colonies producing penicillinase or amidase are hence positive. The results can be confirmed microbiologically as later described by Knox and Smith (1961). A modification has been described (Novick, R.P., personal communication) using *N*-phenyl-1-naphthylamine azocarboxybenzene as indicator; this compound precipitates as it changes to the purple coloured form at acid pH values, and thus diffusion of the dye does not occur: this modification is of use in the study of mixed populations.

Iodometric method. Foley and Perret (1962) have described a method based on the principle of the iodometric assay (Perret, 1954): penicillinase-producing colonies decolorise starch iodine impregnated filter paper, whilst other colonies do not.

Both these methods can be used to "screen" large numbers of organisms for penicillinase activity. In addition, the filter membrane method will indicate the amidase producers.

Haight-Finland (1952) *method.* This procedure, a modification of the method of Gots (1945), is recommended by Woolff and Hamburger (1962). An agar plate containing *Sarcina lutea*, and a just inhibitory amount of penicillin G, is inoculated with the organism under investigation and incubated. Satellite colonies of *S. lutea* grow in the region where penicillinase has destroyed the penicillin G.

The method is time-consuming and uneconomical as only five or six organisms can be investigated on each plate. However, by using a filter

membrane (Knox and Smith, 1961) many colonies of organisms can be investigated simultaneously using the same principle. The organisms are grown overnight on a filter membrane on nutrient agar containing 0·05 μg./ml. of penicillin G. The filter membrane is then discarded and the penicillin G-containing plate is inoculated with a confluent growth of *Sarcina lutea* and incubated. The *Sarcina lutea* then grows in the areas where penicillin G has been destroyed.

Chromatographic methods. Early workers using chromatographic procedures were concerned only with discovering the different number of penicillins present in fermentation liquors; chromatograms can be developed with biological systems (Goodall and Levi, 1946; Winsten and Spark, 1947); Baker, Dobson and Martin (1950) developed a colour reaction—they applied the hydroxamates of penicillins to the paper, ran in isopropyl ether + 15 per cent (w/v) isopropanol saturated with 0·1M potassium hydrogen phthalate, and sprayed with ferric chloride solution. The brown spots were eluted into n-butanol and the extinction read with an EEL colorimeter. Thus both quantitative and qualitative assays were performed simultaneously. The sensitivity of their method was 100 μg. Solvent systems that have been used are: water saturated diethyl ether (Glister and Grainger, 1950); n-butanol 4:ethanol 1:water 5; n-butanol 12:acetic acid 3:water 5; n-butanol 1:pyridine 1:water 1 (Cole and Rolinson, 1961).

Colour reactions, based on the iodometric method, have recently been developed (Thomas, 1961; Sneath and Collins, 1961). 6-Aminopenicillanic acid has been detected by phenylacetylation (Batchelor and others, 1959; Cole and Rolinson, 1961; Uri and Sztaricskai, 1961). Work in this laboratory has shown that ninhydrin used with the n-butanol 4:acetic acid 1:water 5 solvent, or the xylose-aniline spray of Saarnio Niskasaarki and Gustafsson (see Hulme, 1961) with the solvent system n-propanol 6:ammonia 3:water 1 (Hanes and Isherwood, 1949) will detect about 25 μg. of penicillins, penicilloic acids and 6-aminopenicillanic acid, in enzymic hydrolysis mixtures; excellent separation is obtained with both systems. Huang and others (1960) have devised a method for estimating aminopenicilloic acid by means of quantitative paper chromatography using ninhydrin as the colour developer.

Thus chromatographic methods have obvious applications for the differentiation between penicillinases and amidases.

Enzyme Units

Pollock and Torriani (1953) defined the unit of penicillinase as that amount of enzyme that hydrolyses 1 μmole of penicillin G per hr. at pH 7·0 and 30°. The figures were chosen because these workers were using the manometric assay. Other assay methods use different physical conditions, and so other definitions of the enzyme unit have appeared. A commonly used definition quotes pH 7·46 and 37°. (See for instance Leitner and Cohen (1962) who attribute this definition to Pollock and Torriani.) Perret (1954) defines his unit at pH 6·5 and 30°, and Novick (1962a) introduces another set of physical conditions, pH 5·8 and 30°,

and also attributes the definition to Pollock and Torriani. The position hence remains unsatisfactory; a relevant discussion of the experimental conditions and the units that should be used for enzymes is given by Thompson (1962).

Conclusions

Methods for Determining Penicillin Destruction

It has been emphasised that not all methods for estimating penicillin breakdown can be used with all penicillins. The hydrolysis of 6-aminopenicillanic acid by penicillinase cannot be followed either by the acidimetric or the manometric assays; it is better to avoid iodometric methods for penicillins that absorb iodine with their β-lactam ring intact.

By means of a screening technique, such as the filter membrane method, many organisms can be investigated simultaneously for penicillin destruction. In the light of modern developments, it is important to include a test for amidase activity on organisms positive in the filter membrane test, and for this purpose the butyl acetate extraction technique must be used. Furthermore, chromatography at an early stage in the investigation will also elucidate the precise nature of the hydrolytic cleavage of the penicillin. Quantitative amidase estimation can be carried out by the butyl acetate extraction technique or by chromatography, but neither is completely satisfactory for this purpose.

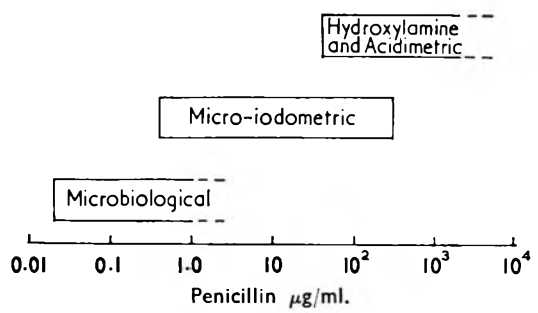


FIG. 1. The relation between the various methods and their use with varying concentrations of penicillins.

Methods for Estimating Penicillins

For assaying minute amounts of penicillins, microbiological methods are the only ones available, and by careful choice of experimental conditions and indicator organism, levels as low as 0·02 μg./ml. of penicillin G can be detected. For higher concentrations of penicillin (for example above 20 μg./ml.) the hydroxylamine assay is the most useful method because it gives a direct estimate of penicillin concentration. All the remaining methods are indirect, since it is necessary first to convert the penicillin to its corresponding penicilloic acid and then estimate this. However, when a chemical method is needed for concentrations less than 20 μg./ml. the iodometric method is the most suitable. Of all the methods

described above, the hydroxylamine and iodometric methods are the most useful for quantitative estimation of penicillins and penicillinase. The hydroxylamine method, being specific for the β-lactam ring, is the method of choice for relatively large concentrations of substrate and penicillinase. The micro-iodometric method is then available for measuring low levels of penicillinase, although it must be borne in mind that it gives results 40 per cent lower than those obtained with other assays. The relation between the various methods and their use with varying concentrations of penicillins is represented diagrammatically in Fig. 1.

Acknowledgement. We would like to thank Mr. F. R. Batchelor for a valuable discussion, and Dr. R. P. Novick for his kind cooperation.

References

Abraham, E. P. (1951). In *The Enzymes*, Vol. 1, ii, Editors Sumner, J. B. and Myrbäck, K., pp. 1170–1185. New York: Academic Press.
Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G. and Jennings, M. A. (1941). *Lancet*, **2**, 177–188.
Abraham, E. P. and Newton, G. G. F. (1956). *Biochem. J.*, **63**, 628–634.
Alicino, J. F. (1946). *Industr. Engng Chem. (Anal. Ed.)*, **18**, 619–620.
Baker, P. B., Dobson, F. and Martin, A. J. P. (1950). *Analyst*, **75**, 651–656.
Batchelor, F. R., Cameron-Wood, J., Chain, E. B. and Rolinson, G. N. (1961). *Proc. roy. Soc.*, B, **154**, 514–521.
Batchelor, F. R., Chain, E. B., Hardy, R., Mansford, K. R. L. and Rolinson, G. N. (1961). *Ibid.*, **154**, 498–508.
Batchelor, F. R., Chain, E. B. and Rolinson, G. N. (1961). *Ibid.*, **154**, 478–489.
Batchelor, F. R., Doyle, F. P., Nayler, J. H. C. and Rolinson, G. N. (1959). *Nature, Lond.*, **183**, 257–258.
Batchelor, F. R., Gazzard, D. and Nayler, J. H. C. (1961). *Ibid.*, **191**, 910–911.
Benedict, R. G., Schmidt, W. H. and Coghill, R. D. (1945). *Arch. Biochem.*, **8**, 377–384.
Boxer, G. E. and Everett, P. M. (1949). *Analyt. Chem.*, **21**, 670–673.
Citri, N. (1958). *Biochim. Biophys. Acta*, **27**, 277–281.
Claridge, C. A., Gourevitch, A. and Lein, J. (1960). *Nature, Lond.*, **187**, 237–238.
Cole, M. and Rolinson, G. N. (1961). *Proc. roy. Soc.*, B, **154**, 490–497.
Colon, A. A., Herpich, G. E., Neuss, J. D. and Frediani, H. A. (1949). *J. Amer. pharm. Ass.*, **38**, 138–142.
Cooper, K. E. (1955). *Nature, Lond.*, **176**, 510–511.
Cooper, K. E. and Linton, A. N. (1952). *J. gen. Microbiol.*, **7**, 8–17.
Cooper, K. E. and Woodman, D. (1946). *J. Path. Bact.*, **58**, 75–84.
Crompton, B., Jago, M., Crawford, K., Newton, G. G. F. and Abraham, E. P. (1962). *Biochem. J.*, **83**, 52–63.
English, A. R., MacBride, T. J. and Huang, H. T. (1960). *Proc. Soc. exp. Biol. N.Y.*, **104**, 547–549.
Ferrari, A., Russo-Alesi, F. M. and Kelly, J. M. (1959). *Analyt. Chem.*, **31**, 1710–1717.
Foley, J. M. and Perret, C. J. (1962). *Nature, Lond.*, **195**, 287–288.
Ford, J. H. (1947). *Indust. Engng Chem. (Anal. Ed.)*, **19**, 1004–1006.
Foster, J. W. (1945). *Science*, **101**, 205.
Foster, J. W. and Woodruff, H. B. (1943a). *J. Bact.*, **46**, 187–202.
Foster, J. W. and Woodruff, H. B. (1943b). *J. biol. Chem.*, **148**, 723.
Foster, J. W. and Woodruff, H. B. (1944). *J. Bact.*, **47**, 43–58.
Glister, G. A. and Grainger, A. (1950). *Analyst*, **75**, 310–314.
Goodall, R. R. and Davies, R. (1961). *Ibid.*, **86**, 326–335.
Goodall, R. R. and Levi, A. A. (1946). *Nature, Lond.*, **158**, 675–676.
Gots, J. J. (1945). *Science*, **102**, 309.
Green, N. C. and Monk, P. R. (1959). *Chem. and Ind.*, 1210–1214.
Grove, D. C. and Randall, W. A. (1955). In *Assay Methods of Antibiotics*, Medical Encyclopaedia Inc., pp. 16.
Haight, T. H. and Finland, M. (1952). *Amer. J. clin. Path.*, **22**, 806–808.
Hanes, C. S. and Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107–1112.
Heatley, N. G. (1944). *Biochem. J.*, **38**, 61–65.

Heatley, N. G. (1948). *Analyst*, **73**, 244–247.
Henry, R. J. and Housewright, R. D. (1947). *J. biol. Chem.* **167**, 559–571.
Henstock, H. I. (1949). *Nature, Lond.*, **164**, 139–140.
Hiscox, D. J. (1949). *Analyt. Chem.*, **21**, 658–659.
Huang, H. T., English, A. R., Seto, T. A., Shull, G. M. and Sobin, B. A. (1960). *J. Amer. chem. Soc.*, **82**, 3790–3791.
Hulme, A. C. (1961). *Adv. applied Microbiol.*, **3**, 343–393.
Jeffrey, J. d'A., Abraham, E. P. and Newton, G. G. F. (1961). *Biochem. J.*, **81**, 591–596.
Kavanagh, F. (1960). *Adv. applied Microbiol.*, **2**, 65–93.
Knox, R. and Smith, J. T. (1961). *Nature, Lond.*, **191**, 926–927.
Knox, R. and Smith, J. T. (1962). *J. gen. Microbiol.*, **28**, 471–479.
Lees, K. A. and Toothill, J. P. R. (1955). *Analyst*, **80**, 95–110.
Leitner, F. and Cohen, S. (1962). *J. Bact.*, **83**, 314–323.
Lipmann, F. and Tuttle, L. L. (1945). *J. biol. Chem.*, **159**, 21–28.
McKee, C. M., Rake, G. and Menzel, A. E. O. (1944). *J. Immunol.*, **48**, 259–270.
Manson, E. E. D., Pollock, M. R., and Tridgell, E. J. (1954). *J. gen. Microbiol.*, **11**, 493–505.
Murao, S. (1955). *J. Agric. Chem. Soc. Japan*, **29**, 400–403.
Murtaugh, J. J. and Levy, G. B. (1945). *J. Amer. chem. Soc.*, **67**, 1042.
Niedermayer, A. D., Russo-Alesi, F. M., Lendzian, C. A. and Kelly, J. M. (1960). *Analyt. Chem.*, **32**, 664–666.
Novick, R. P. (1962a). *Biochem. J.*, **83**, 229–235.
Novick, R. P. (1962b). *Ibid.*, **83**, 236–240.
Pan, S. C. (1954). *Analyt. Chem.*, **26**, 1438–1444.
Patterson, S. J. and Emery, W. B. (1948). *Analyst*, **73**, 207–211.
Perret, C. J. (1954). *Nature, Lond.*, **174**, 1012–1013.
Pollock, M. R. (1952). *Brit. J. exp. Path.*, **33**, 587–600.
Pollock, M. R. (1961). *J. gen. Microbiol.*, **26**, 239–253.
Pollock, M. R. and Torriani, A. M. (1953). *C.R. Acad. Sci. (Paris)*, **237**, 276–278.
Rolinson, G. N., Batchelor, F. R., Butterworth, D., Cameron-Wood, J., Cole, M., Eustace, G. C., Hart, M. V., Richards, M. and Chain, E. B. (1960). *Nature, Lond.*, **187**, 236–237.
Rolinson, G. N. and Stevens, S. (1961). *Proc. roy. Soc.*, B, **154**, 509–513.
Rollo, I. M., Somers, G. F. and Burley, D. N. (1962). *Brit. med. J.*, **1**, 76–80.
Saz, A. K., Lowery, D. L. and Jackson, L. J. (1961). *J. Bact.*, **82**, 293–304.
Scudi, J. V. (1946). *J. biol. Chem.*, **164**, 183–194.
Scudi, J. V. and Jellinek, V. C. (1946). *Ibid.*, **164**, 195–201.
Schmidt, W. K. and Moyer, A. J. (1944). *J. Bact.*, **47**, 199–208.
Sheehan, J. C., Henery-Logan, K. R. and Johnson, D. A. (1953). *J. Amer. chem. Soc.*, **75**, 3292–3293.
Sneath, P. H. A. and Collins, J. F. (1961). *Biochem. J.*, **79**, 512–514.
Staab, F. W., Ragan, E. A. and Binkley, S. B. (1946). Abs. 109th meeting Amer. Chem. Soc. 3 B.
Steinman, H. (1961a). *Proc. Soc. exp. Biol., N.Y.*, **106**, 227–231.
Steinman, H. (1961b). *Antibiotics and Chemotherapy*, **11**, 537–542.
Thomas, R. (1961). *Nature, Lond.*, **191**, 1161–1163.
Thomson, R. H. S. (1962). *Ibid.*, **193**, 1227–1231.
Tucker, R. G. (1954). *Ibid.*, **173**, 85.
Twigg, G. H. (1948). *Analyst*, **73**, 211–214.
Uri, T. and Sztarcskai, F. (1961). *Nature, Lond.*, **191**, 1223.
Weiss, P. J. (1959). *Antibiotics and Chemotherapy*, **9**, 660–666.
Winsten, W. A. and Spark, A. H. (1947). *Science*, **106**, 192–193.
Woolff, D. A. and Hamburger, M. (1962). *J. Lab. clin. Med.*, **59**, 469–480.

RESEARCH PAPERS

THE ASSESSMENT OF CONDUCTION ANAESTHESIA

By G. C. Jefferson*

From the Department of Pharmacology, Manchester University

Received July 30, 1962

Experiments are described which involve observation of the rates of decline in the compound action potentials of frog sciatic nerve produced by local anaesthetics. The percentage reduction and regression, with time, of action potentials are assessed for each of four concentrations of lignocaine hydrochloride. The results are analysed for variance. It appears that percentage reduction of action potential gives a better assessment of conduction anaesthesia than the regression of potential with time. The percentage reduction of action potential bears a linear relationship to the logarithm of the concentration of local anaesthetic applied to the nerve.

Conduction anaesthesia has been considered to involve the penetration of nerve sheaths by anaesthetic agents, resulting in reversible paralysis of the nerve (see Sollmann, 1917; Sinha, 1936; Bülbring and Wajda, 1945). The isolated frog sciatic nerve has become accepted as a standard preparation for its measurement since the experiments of Bennett, Wagner and McIntyre (1942).

In the present work it was thought that the measurement of the action potentials of such nerves, as absolute voltages, would be a direct assessment of activity in a given nerve trunk. By observation of the extent of changes effected in the potentials by local anaesthetic, it was hoped to investigate the relationship between the concentration of drug applied and the conduction anaesthesia produced.

Experimental

Preparation of the Nerve

Sciatic nerves of pithed frogs (*Rana temporaria*) of either sex were dissected under frog Ringer solution (as modified by Starling) from the ninth root to the knee, each end being tied with silk thread. They were then stored overnight (approximately 16 hr.) at 5°, allowing the nerves to stabilise after any injury during dissection. The frogs were not less than 25 g. in weight, giving a length of nerve of 4 to 6 cm.

Apparatus

The apparatus consisted of an enclosed cabinet, the front of which could be raised. In the cabinet, suspended from a perspex beam (B), were two stimulating (SS), one earth (E) and two recording electrodes (R′R′) (Fig. 1), all of 20 s.w.g. platinum wire. The recording electrodes which were at a fixed distance apart (12 mm.), could be adjusted relative to the

* Present address: Department of Pharmacy, The Heriot-Watt College, Edinburgh.

other electrodes in order to accommodate any variation in the lengths of nerves used.

A notch (N) in one end of the perspex beam and a pulley system (P′P″) at the other end allowed the nerve, ligatured at both ends, to be held in position over the electrodes, aided by tension applied to the nerve from a 1 g. weight. This prevented the position of the nerve on the electrodes from changing during application of the drug, or washing.

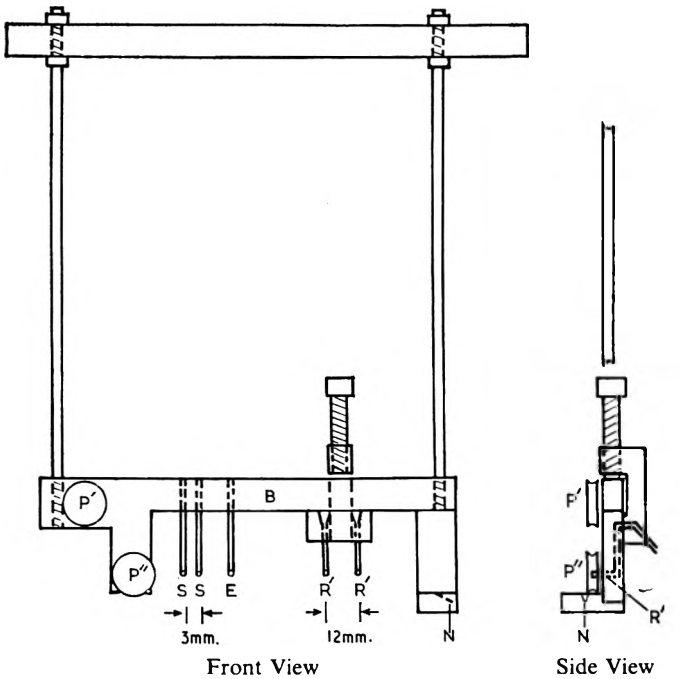


FIG. 1. Arrangement of electrodes in apparatus. B, Perspex beam. SS, Stimulating electrodes. R′R′, Recording electrodes. E, Earth electrode. Pulley system (P′P″) and notch (N) allowed nerves, ligatured at both ends, to be held in position over the electrodes.

A perspex trough, volume 45 ml., by means of which the nerve could be washed with Ringer solution, and a perspex cup for the anaesthetic, volume 0·3 ml., were incorporated in the enclosed cabinet. Both could be manipulated from outside the cabinet. The cup had V-shaped notches cut in opposing faces. This enabled the nerve to pass through the meniscus of a constant volume of anaesthetic solution, which therefore surrounded the nerve without the nerve touching the sides of the cup.

In the cabinet, humidity was maintained at saturation throughout experiments by means of (15 cm. × 30 cm.) chromatographic paper suspended from beams at either end of the cabinet, dipping into perspex troughs containing water. Humidity, which was measured by a paper hygrometer, was initially raised to saturation by blowing water vapour

into the cabinet. The temperature of the cabinet was raised to, and maintained at, 24° by table and overhead heaters.

After placing the nerve in position, the stimulating electrodes were connected with a stimulator, the recording electrodes were connected with the input of the recording apparatus and the fifth electrode was led to earth. All leads were "screened".

Electronic apparatus. A block diagram of this apparatus is given in Fig. 2. The rate of firing of the stimulator and of the sweep generator was controlled by the master oscillator. After delays imposed by the

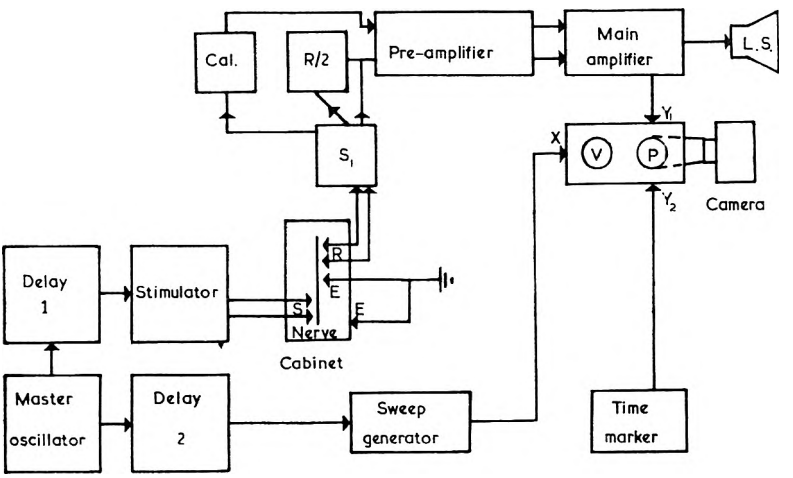


FIG. 2. Block diagram of apparatus. S, Stimulating electrodes. R, Recording electrodes. E, Earth electrodes. S_1, 4-pole, 3-way switch. R/2, Variable parallel resistance. Cal, Calibrator. L.S., Loud speaker. X, Y_1, Y_1, Plates of double beam cathode ray tubes V and P.

delay circuits (1 and 2), the stimulator and sweep generator were triggered. Normally delay 2 was in the zero position so that the traverse of the spot across cathode ray screens V and P began immediately after the firing of the master oscillator. The time at which the stimulus fell was governed by the setting of delay 1. The stimuli were square wave voltage pulses of 0·1 msec. duration, their amplitude being variable between 0 and 12 V. When stimulating the nerves, a pulse just supramaximal for the action potential was delivered every 10 sec.

The potentials picked off from a stimulated nerve were led to a 4-pole, 3-way switch (S_1) and passed to a pre-amplifier by twin screened cables either directly or after passing a variable parallel resistance R/2. The switch also permitted a calibrator (cal.) to be connected with the pre-amplifier. The main voltage amplification was performed in the second or main amplifier. The output of this amplifier was connected to one of the Y-plates of a double beam cathode ray tube and to a small audio-amplifier which fed a loud speaker (L.S.). The latter provided an aural indication of the nervous activity. The other Y-plate of the cathode ray

tube was connected with a time marker giving pulses of 0·1, 1·0 or 10 msec. intervals so that the recorded phenomena could be timed accurately.

The display unit consisted of two double beam flat faced cathode ray tubes, each presenting the same information. One tube (V) was used for visual examination of the trace; the other (P) was attached by a metal cone to a camera in which traces were recorded on 35 mm. film, negative voltages being recorded upwards.

The calibrator was a device whereby a one mV pulse of varying duration could be controlled manually. The voltage was obtained by dividing off the required potential by an appropriate resistance from a 1·5 V cell. The value of the mV was controlled by regulating the current flowing through a resistance of one ohm to one mA.

The calibrator made it possible to assess, in terms of volts, the values of the action potentials observed on the face of the oscillograph screen. This assessment involved recording the Y-plate deflection achieved by the mV pulse on the oscillograph screen.

Thus the value of the standard mV could be determined directly, irrespective of the amplification used in any particular experiment.

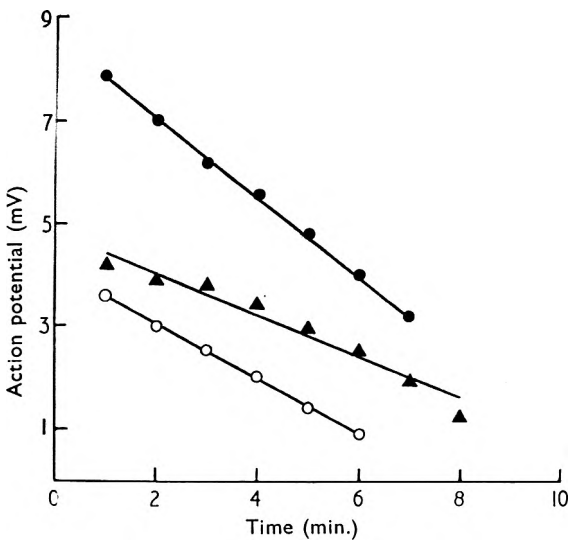


FIG. 3. Decline in action potential in three frog sciatic nerves, following the application of 5 mM lignocaine hydrochloride.

Experimental Procedure

The sciatic nerve was mounted and its action potential rendered monophasic by burning. The time marker was photographed to serve as future reference for temporal estimates. The sweep speed having been adjusted remained set throughout the experiment. The stability of the preparation was tested by recording twenty control action potentials at 10 sec. intervals after adjusting the shock strength of the stimulus. The

drug in the anaesthetic cup was brought up to surround the nerve completely at the point of application and the time was noted. Recording continued until the action potential was reduced to 80 to 90 per cent of its original value when the anaesthetic was removed and the time again noted. The nerve was allowed to rest, surrounded by Ringer solution and the degree of recovery at subsequent 15 min. intervals was ascertained until nearly complete.

Results

The effects of four different concentrations of lignocaine hydrochloride (10, 5, 2·5 and 1·25 mM) were recorded on the action potentials of four different groups of sciatic nerves. Each determination at a given concentration involved a separate nerve, the voltage being measured with the aid of a calibrator.

TABLE I

Effect of lignocaine hydrochloride on the action potential of frog sciatic nerve, measured as the regression (b) in mV/min., or as the "percentage reduction" (p) of potential. p is expressed in min.$^{-1}$ × 100

Concentration (mmolar)	1·25	2·50	5·00	10·00	Ringer
pH	7·20	7·15	7·10	6·90	7·30
Number of determinations	16	16	15	14	8
Mean value of b	0·15	0·43	0·53	0·84	0·04
Variance of b	0·0062	0·0150	0·0970	0·1112	0·03
Mean value of p	2·84	6·68	10·69	15·66	—
Variance of p	3·58	2·97	5·46	9·01	—

As the local anaesthetic blocked conduction the action potential was found to decline linearly with time (Fig. 3). It was thought that the criterion of the effectiveness of a drug as a conduction anaesthetic could be taken as the slope of the line relating voltage and time for a given nerve. The slope was expressed as a regression coefficient (b) in mV/min., the line having been calculated by regression analysis (Saunders and Fleming, 1957). The mean value of b was determined for each concentration of drug used and the variance (S^2) of the observations about each mean value was determined (Table I). Ringer controls are included in this Table.

TABLE II

Correlation coefficients (r) of the mean control action potentials with the mean rates of decline in potential produced by lignocaine hydrochloride

Concentration (mM)	1·25	2·50	5·00	10·0
Number of determinations (N)	16	16	15	14
r_e—calculated	0·02	0·53	0·92	0·84
r_t—theoretical	0·50	0·50	0·52	0·54
N–2 degrees of freedom	—	—	—	—
P = 0·95	—	—	—	—

For significant correlation r_e should be greater than r

However, for each concentration of drug, the mean value of b has been correlated with the mean value of the control action potentials recorded from the nerves (Table II). From Table II it may be seen that at the 10, 5 and 2·5 mM concentrations such a correlation was significant.

Therefore it was decided also to determine for each nerve the "percentage reduction" (p) in action potential as given by the fraction:

$$p = \frac{b}{\text{Initial Action Potential}} \times 100.$$

Thus, p was a measure of the fall-off of potential in unit time as related to the control action potential. However as b, for a given nerve, may be measured by dividing the control action potential by the time taken by the drug to produce a 100 per cent block in conduction, then p can be considered as being a measure of the reciprocal of "100 per cent block time". The variances of p about the mean for each concentration of lignocaine were determined (Table I).

An estimate of whether b or p gave the best measure of conduction anaesthesia was required. Hence, it was necessary to compare the variance of results calculated by these different means at each concentration of lignocaine.

For reproducible results it was desirable that the "spread" of results about a mean should be minimal. As the "spread" is proportional to the square root of the variance, $\sqrt{S^2}$/mean value of b should be a minimum.

To compare regression with "percentage reduction", the null hypothesis that there was no difference in the Variancy ratio $\frac{S^2b/\text{mean}^2b}{S^2p/\text{mean}^2p}$ was made. The hypothesis was tested by determining whether or not the ratio lay within the range 0·38–2·60 as indicated by the "F value" derived from tables for n–1 degrees of freedom at a probability level of 95 per cent.

TABLE III

VARIANCY RATIOS OF REGRESSION (b) AND PERCENTAGE REDUCTION (p) FOR ACTION POTENTIALS IN NERVES TESTED WITH LIGNOCAINE HYDROCHLORIDE

Concentration (mM)	1·25	2·50	5·00	10·0
Number of determinations	16	16	15	14
Variancy ratio $\frac{S^2bv/\text{mean}^2bv}{S^2pv/\text{mean}^2pv}$	0·64	1·22	7·31	4·25

For there to be no significant difference in the variances of regression and "percentage reduction", the Variancy ratio should lie within the range 0·38–2·60. Values greater than 2·60 show that regression has a larger variance.

The results (Table III) obtained suggested that at the 10 and 5 mM concentration p and b are significantly different, the "percentage reduction" of action potential giving less "spread" or variance than regression.

The "percentage reduction" also bears a linear relationship to the logarithm of the concentration of drug applied (Fig. 4). Hence this measure would appear to be preferable in assessing conduction anaesthesia.

Discussion

One of the most sensitive devices for the study of activity in a nerve trunk was introduced by the application of the cathode ray oscillograph to electro-physiological work by Gasser and Erlanger in 1922. As local anaesthesia involves a reversible block of nerve conduction it was to be

expected that some of the effects of local anaesthetics on nervous tissue could be observed and possibly assessed from oscillographic recordings. Such assessments were in fact attempted by Bennett and others (1942) and Bennett and Chinburg (1946). The limitations of this approach to the screening and bioassay of local anaesthetics seemed to lie in the fact that the method can be applied only to conduction anaesthesia.

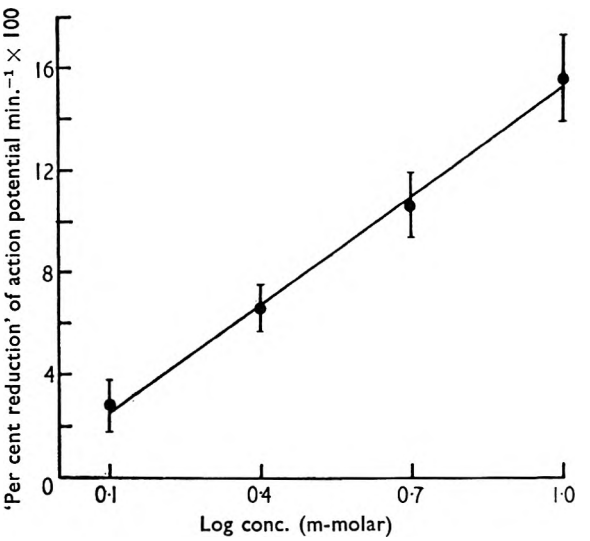


FIG. 4. "Percentage reduction" of action potential produced by lignocaine hydrochloride in frog sciatic nerve: mean value ● and limits of error of mean. The line relating "percentage reduction" and log concentration was determined by regression analysis (Saunders and Fleming, 1957).

The work of Bennett and others (1942) involved the use of isolated frog sciatic nerve, a mixed nerve trunk, whereas ideally the effectiveness of local anaesthetics should be assessed on sensory nerve fibres alone. Indeed, as early as 1917 Sollmann had criticised the use of motor nerve fibres in assessing conduction anaesthesia. The use of frog sciatic nerve in such studies has, however, persisted. Results obtained from such experiments are less subjective than those obtained from methods involving intracutaneous injection (as in infiltration anaesthesia) or surface anaesthesia which involve the study of reactions of whole animals (Sinha, 1936).

The stimulus for the present work lay in the fact that the action potential might give a measure of the number of active fibres in a nerve trunk. It has been found that an assessment of conduction anaesthesia could be made which would be capable of statistical analysis. A compound action potential has been the basic measurement in this work. It has not been possible to examine the effects of lignocaine on its possible subdivisions since such resolution requires nerves rather longer than those which were obtained from *R. temporaria.*

The action potential was found to fall off in a linear manner as the nerve fibres were progressively blocked by local anaesthetic. As this regression, in a given nerve, was related to the control action potential, it was decided to compare results obtained by measuring regression of potential with those obtained from determinations of the "percentage reduction" of potential.

"Percentage reduction" may be considered as a function of the time required by a concentration of drug to produce a 100 per cent block of conduction in a given nerve. It appeared to give more reproducible results than measures of regression.

The "percentage reductions" have been expressed as a dose-response relationship which has been applied for the first time to results obtained from work in conduction anaesthesia involving observations on action potentials. It is established that different concentrations of anaesthetics produce rates of nerve blockade which are significantly different.

Sensitivity of the method is seen from the fact that preparations presented stable potentials during control periods, whilst the smallest concentrations of drug exerted a noticeable effect on these potentials. Similarly there was a difference between the results obtained when a low concentration of drug, and when Ringer solution, was applied to the nerve.

It should be noted that the method employed in the present work, unlike that of Bennett and Chinburg (1946) does not give any indication of the mechanism by which the nerve block is produced. They were able to investigate the mechanism of nerve block by local anaesthetics from a study of resting and demarcation potentials in isolated frog sciatic nerve.

Ultimately, it is likely that the problems of the site and mechanism of action of local anaesthetics on peripheral nerve will be resolved. Much knowledge of these subjects could be gained by a study of stereochemically related substances if their anaesthetic potencies could be compared accurately.

Thus, the present work has described an attempt to provide an objective and reliable method for the quantitative biological comparison of substances which may have local anaesthetic qualities.

Acknowledgements. The author wishes to record his grateful thanks to Dr. F. B. Beswick of the Department of Physiology, Manchester University, for help and advice and the Pharmaceutical Society of Great Britain for financial support.

References

Bennett, A. L. and Chinburg, K. G. (1946). *J. Pharmacol.*, **88**, 72–81.
Bennett, A. L., Wagner, J. C. and McIntyre. A. R. (1942). *Ibid.*, **75**, 125–136.
Bülbring, E. and Wajda, I. (1945). *Ibid.*, **85**, 78–84.
Gasser, H. S. and Erlanger, J. (1922). *Amer. J. Physiol.*, **62**, 496–524.
Saunders, L. and Fleming, R. (1957). *Mathematics and Statistics for use in Pharmacy, Biology and Chemistry*, p. 157. London: The Pharmaceutical Press.
Sinha, H. K. (1936). *J. Pharmacol.*, **57**, 199–220.
Sollmann, T. (1917). *Ibid.*, **10**, 379–397.

THE DETERMINATION OF POLDINE METHYL METHOSULPHATE IN BIOLOGICAL FLUIDS

By P. F. Langley, J. D. Lewis, K. R. L. Mansford and D. Smith

From the Biochemistry Department, Beecham Research Laboratories Limited, Brockham Park, Betchworth, Surrey

Received July 30, 1962

A spectrophotometric method is described for the quantitative determination of the quaternary ammonium compound, poldine methyl methosulphate in aqueous solution and in biological fluids. The method involves coupling with methyl orange with simultaneous extraction into a mixture of ethylene dichloride and isopentanol and the determination of the optical density of the resulting acidified solution. The effect of pH on the coupling and extraction and the specificity of the reaction towards related compounds are examined.

Following successful clinical trials (Douthwaite, Hunt and Macdonald, 1957; Douthwaite and Hunt, 1958) as a gastric secretion inhibitor, the distribution, metabolism and rate of excretion of poldine methyl methosulphate (Nacton) were studied.

Published methods for the analysis of quaternary nitrogen compounds were all unsuitable when applied to the determination of this compound in biological fluids.

The bromophenol blue method of Levine and Clark (1955) was too insensitive and attempted coupling under acid and alkaline conditions with other sulphone-phthalein indicators followed by solvent extraction was not successful. The ammonium cobaltothiocyanate (Helgren, Theivagt and Campbell, 1957; Singleton and Wells, 1960), bromothymol blue (Helgren and others, 1957) and the bromocresol purple methods (Fogh, Rasmussen and Skadhaugl, 1954) were also investigated, but were again too insensitive. An examination of the chloranil reaction and the aconitic anhydride reaction of Sass, Kaufman, Cardenas and Martin (1958) revealed some interesting differences in the reactions of poldine and similar compounds, but no quantitative method of sufficient sensitivity resulted.

The method finally adopted for use in distribution and excretion studies was based on that suggested by Brodie and Udenfriend (1945) in which simultaneous coupling with methyl orange and extraction into an organic solvent is used, followed by spectrophotometric measurement after acidification.

Experimental

Reagents

Ethylene dichloride. Reagent grade ethylene dichloride was washed successively with N Analar NaOH (2 × ⅕ vol.), distilled water (2 × ⅕ vol.), N Analar HCl (2 × ⅕ vol.) and then 6 times with distilled water. It was then filtered through absorbent cotton wool to remove water. The ethylene dichloride was purified every 4 weeks.

Isopentanol. This was washed in the same manner as the ethylene dichloride and then fractionally distilled. After removal of the water, the clear distillate (b.p. 130–132°) was used.

Methyl orange reagent. Solid methyl orange was exhaustively extracted with ethylene dichloride in a Soxhlet extractor and then a 0·5 per cent w/v solution in water was made up. Immediately before use, equal volumes of this aqueous methyl orange solution and saturated aqueous boric acid were mixed. The mixed solution had a limited stability and was not used when turbidity had appeared.

Ethanolic sulphuric acid. Conc. H_2SO_4 (2 ml. S.G. 1·84) in ethanol (100 ml.) 5N-*Hydrochloric acid* (Analar grade).

Dipotassium hydrogen phosphate. Reagent grade (50 per cent) aqueous solution stored at +4°.

Determination of Poldine in Aqueous Solutions*

To 4 ml. of the test solution or of poldine standard solution normally containing 25–100 μg. were added ethylene dichloride (10 ml. containing 5 per cent v/v isopentanol, hereafter called EDC reagent), followed by methyl orange reagent (0·5 ml.). After manual shaking for 2 min. in a glass-stoppered tube, the mixture was centrifuged at about 700 *g*. The top aqueous layer was removed by aspiration. Any residual water droplets containing methyl orange were removed by filtration of the lower EDC layer into a dry test tube. 5 ml. of the filtrate was pipetted into another dry test tube, followed by ethanolic H_2SO_4 (1 ml.). After thorough mixing of the added acid, the resulting pink colour was read in a 2 cm. glass cell of the Unicam SP600 at 525 mμ against a blank, using distilled water initially in place of the test or poldine standard solution.

If the colour was too dense to read, the solution was suitably diluted with EDC reagent. Beer's law was then obeyed up to 1000 μg. poldine.

When analysing unknown solutions for poldine, standards were performed simultaneously since there was a small but appreciable variation in standard graphs prepared on different days.

Determination of Poldine in Urine

The pH of the urine was adjusted to 7·5 by the addition of 50 per cent K_2HPO_4. After measuring the volume, it was centrifuged at about 2000 *g* for 10 min. to remove any solid present. 4 ml. of supernatant was added to a glass-stoppered tube containing water or standard poldine solution (4 ml.), EDC reagent (10 ml.) and methyl orange reagent (0·5 ml.). The procedure was then as for the determination of poldine in aqueous solutions.

Determination of Poldine in Tissue

Preparation of tissue. After the removal of fat and all connective tissue, the organs were briefly rinsed in distilled water, blotted dry and

* In the text the approved name refers to the methyl methosulphate except where stated otherwise.

weighed. They were then homogenised in an M.S.E. homogeniser with an equal weight of water and transferred to a measuring cylinder. The homogeniser blades and tissue container were rinsed with water and the washings added to the homogenate. The homogenate was diluted to give a volume numerically three times the weight of the tissue and 3 ml. of homogenate (i.e. 1 g. tissue) was used for each determination. The consistency of this homogenate was such that it could be pipetted using a wide jet pipette. It was found that the alimentary tract was more difficult to homogenise but by grinding the frozen tissue after cutting into small pieces and then using the above procedure, a satisfactory homogenate could be obtained.

Method of deproteinising. 3·0 ml. homogenate prepared as above (i.e. 1·0 g. tissue) was heated in a boiling water-bath for 30 min. with 1·0 ml. 5N HCl and 5·0 ml. water or poldine standard solution. A glass bulb was used to prevent evaporation from the tube. The mixture was then rapidly cooled and transferred to a 15 ml. centrifuge tube and centrifuged for 10–15 min. at about 1000 g. An aliquot of the clear supernatant (5·0 ml.) was added to a glass-stoppered tube containing aqueous K_2HPO_4 solution (4 ml.), EDC reagent (10 ml.) and methyl orange reagent (0·5 ml.). These quantities gave a pH of 7·2. The extraction, coupling and reading procedures were as described above.

Determination of Poldine in Blood

The method described for tissues was equally suitable for the determination of poldine in blood. 1·0 ml. blood was used instead of 3·0 ml. homogenate and after removal of protein, 4·0 ml. of the supernatant was taken.

RESULTS

Stability of Methyl Orange Complex

Both the acidified and non-acidified forms of the methyl orange complex in EDC reagent are stable for at least 24 hr. at $+4°$. After a week the optical density of an acidified solution changed only from 0·87 to 0·91.

Reproducibility and Stability in Homogenates

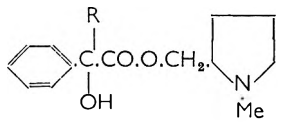
A standard poldine solution was added to a rat liver homogenate which was stored at $-17°$. Daily determinations over a period of 7 days showed that the recovery of poldine was $99{\cdot}2 \pm 1{\cdot}8$ per cent (mean $\pm$ s.e.m.).

Efficiency of Extraction

A comparison of the standard graph obtained from aqueous poldine solutions with a standard graph of poldine in EDC reagent showed that the efficiency of the extraction of the methyl orange-poldine complex was 89 per cent over the range 25–100 μg. poldine. Investigation of variation in the shaking time showed that extraction was completed within 1 min.

Specificity of Methyl Orange Coupling Method

The specificity of the methyl orange coupling reaction was investigated in a series of compounds based on poldine.



I

Using the method described for aqueous solutions, it was found that the tertiary esters, (1-methyl-2-pyrrolidyl)methyl benzilate (poldine base) (I, R = Ph) and (1-methyl-2-pyrrolidyl)methyl α-cyclohexylmandelate (I, R = cyclohexyl), both couple satisfactorily with methyl orange. However, the resultant standard graphs of optical density against concentration show a slight deviation from Beer's law. The sensitivities of poldine base and methyl methosulphate and the methyl methosulphate of I (R = cyclohexyl) are very similar but that of the latter base is considerably less.

N-methyl prolinol is comparatively insensitive to methyl orange coupling and *N*-methylprolinol methyl methosulphate is completely insensitive.

The Effect of HCl Treatment on the Recovery of Poldine from Rat Liver Homogenate, Faeces and Urine

The HCl method was the standard procedure for the determination of poldine in tissue homogenates. The method without HCl treatment used 3·9M sodium chloride (1·0 ml.) in place of the HCl and the aliquot of the supernatant (5·0 ml.) was added to the phosphate buffer pH 7·4 (4 ml.; 50 per cent) in place of the aqueous K_2HPO_4 solution to retain the same molar salt concentration at the coupling stage. For an aqueous comparison, water (3·0 ml.) was substituted for the 3·0 ml. of homogenate.

With liver, faeces and urine the standard graphs showed that different amounts of complex were obtained (see Fig. 1). Taking the recovery of poldine in the absence of tissue under these conditions as 100 per cent, the recoveries from liver homogenate in the presence and absence of HCl were 89 and 68 per cent respectively; from faeces the recoveries were 91 and 80 per cent and from urine they were 95 and 88 per cent. These figures stress the importance of the HCl treatment for the determination of poldine in tissues.

Effect of pH on the Coupling Reaction

The pH range of coupling was investigated by the aqueous method modified so that the molar salt concentration was made the same as in the HCl deproteinising method.

Sodium dihydrogen phosphate and dipotassium hydrogen phosphate with varying volumes of hydrochloric acid and sodium hydroxide solution were used to obtain the different pH values at equal molarities.

From Fig. 2 it can be seen that the range of pH for efficient coupling was between pH 4·0 and 9·0. Above pH 9·0 and below pH 4·0 the colour yield fell off very sharply.

pH Stability

Under strongly acid or strongly alkaline conditions no extraction of poldine by EDC reagent occurred nor was it possible to couple and extract simultaneously under these conditions. Further qualitative experiments showed that on making a poldine solution strongly alkaline and then adjusting the pH to about 6·5 and attempting to couple, no coloration was produced. However, if the solution were made strongly acid and then the pH readjusted to about 6·5, coupling did occur.

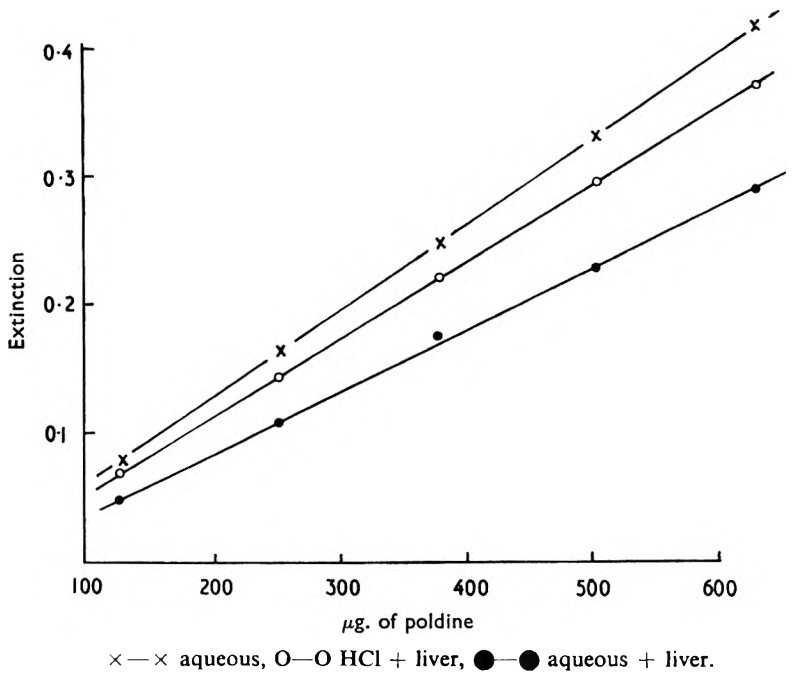


FIG. 1. The effect of HCl treatment on the recovery of poldine from liver homogenates.

DISCUSSION

As poldine is decomposed at a high pH and is insoluble in light petroleum and only sparingly soluble in ethylene dichloride, chlorcform, carbon tetrachloride or benzene, the method of Brodie, Udenfriend and Baer (1947) required modification. The complex formed between methyl orange and poldine is, however, soluble in chloroform and ethylene dichloride and the latter was found to be slightly better for extraction of the complex.

The experiments on the recovery of poldine from rat liver homogenate, faeces and urine showed that a proportion of the poldine was not being determined by the ordinary aqueous method in the presence of tissue or faecal homogenate, or urine. The analyses with HCl present resulted in increased coupling but still not to the extent of that obtained in aqueous solution. It is thus apparent that the tissue is either destroying the poldine, or is affecting the extraction. As standards are performed with all tests, the quantitative determinations should not be affected.

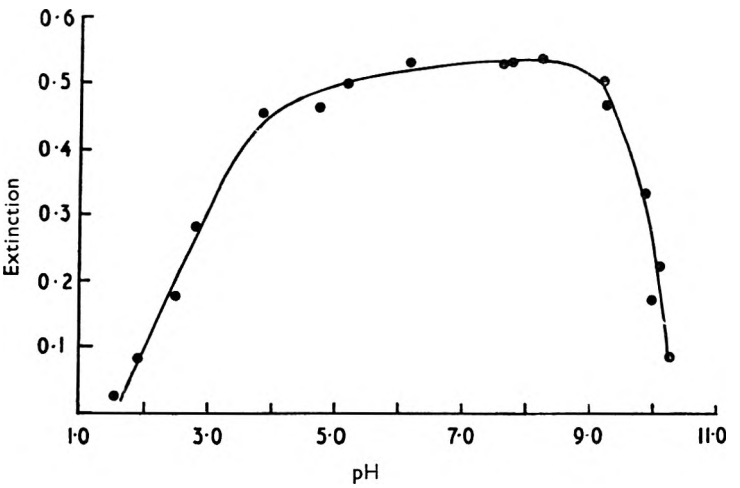


FIG. 2. The effect of pH on the coupling of poldine with methyl orange and on the simultaneous extraction with EDC reagent.

In view of the fact that poldine base and methyl methosulphate and *N*-methyl prolinol all couple to some extent with methyl orange and the coupling product can be simultaneously extracted into the EDC reagent, it is not possible by this method to establish whether the compound assayed in the tissue distribution and excretion studies is in fact the unchanged quaternary compound. Although the base has a similar sensitivity towards coupling as the methyl methosulphate, a metabolite may differ in its sensitivity.

The fact that poldine couples well with methyl orange whereas *N*-methyl prolinol methyl methosulphate does not couple at all, shows that the benzilic acid moiety has some influence on the coupling reaction. This can be compared with the work of Singleton and Wells (1960), who found that whereas poldine couples with ammonium cobaltothiocyanate, *N*-methyl prolinol methyl methosulphate does not react with this reagent.

Acknowledgements. The authors wish to thank Miss Jean Freckelton and Mr. J. R. Le Pelley for skilled technical assistance, and Dr. M. Mehta and his colleagues in the Organic Department for the preparation of the compounds.

REFERENCES

Brodie, B. B. and Udenfriend, S. (1945). *J. biol. Chem.*, **158**, 705–714.
Brodie, B. B., Udenfriend, S. and Baer, J. E. (1947). *Ibid.*, **168**, 299–309.
Douthwaite, A. H., Hunt, J. N. and Macdonald, I. (1957). *Brit. med. J.*, **2**, 275–276.
Douthwaite, A. H. and Hunt, J. N. (1958). *Ibid.*, **1**, 1030–1034.
Fogh, J., Rasmussen, P. O. H. and Skadhauge, K. (1954). *Analyt. Chem.*, **26**, 392–395.
Helgren, P. F., Theivagt, J. G. and Campbell, D. J. (1957). *J. Amer. pharm. Ass., Sci. Ed.*, **46**, 639–643.
Levine, R. M., and Clark, B. B. (1955). *J. Pharmacol.*, **114**, 63–77.
Sass, S., Kaufman, J. J., Cardenas, A. A. and Martin, J. J. (1958). *Analyt. Chem.*, **30**, 529–531.
Singleton, D. O. and Wells, G. M. (1960). *J. Pharm. Pharmacol.*, **12**, 171–175*T*.

SUBSTITUTED DITHIOCARBAMATES AND RELATED COMPOUNDS AS TRICHOMONACIDES

By R. M. Michaels, L. J. Peterson and G. L. Stahl

From the Department of Infectious Diseases, The Upjohn Co., Kalamazoo, Michigan, U.S.A.

Received July 3, 1962

A series of derivatives of dithiocarbamic acid and related compounds have been examined against *Trichomonas vaginalis* and *Trichomonas foetus in vitro* and *in vivo* in the mouse and the rat. The most active compound *in vitro*, sodium dimethyldithiocarbamate, caused a 90 per cent inhibition of growth of *T. vaginalis* and *T. foetus* at 0·07 μg./ml. The *in vitro* activity of the other compounds ranged from 0·17–1·5 μg./ml. There was no direct correlation of *in vitro* and *in vivo* activity. In the assay in mice, sodium 4-morpholinecarbodithioate was the most active compound against *T. vaginalis*. The CD 50 was 3·9 mg./kg. This compound would not cure rats infected intravaginally with *T. vaginalis*. *In vitro* there was an area of non-response in the dose-response curve of *T. vaginalis* to sodium 4-morpholinecarbodithioate and piperidine 1-piperidinecarbodithioate. When mice were infected with *T. vaginalis*, the infections were completely susceptible to treatment with low doses of sodium 1- pyrrolidinecarbodithioate and bis(dimethylthiocarbamoyl) sulphide, but not higher doses. Both of these phenomena, *in vitro* and *in vivo*, may have been due to differences in the solubility of various metal complexes formed by these compounds.

Certain *N*-substituted derivatives of dithiocarbamic acid and of its oxidation product bis(thiocarbamoyl) disulphide are bacteriostatic and fungistatic (Chabrier, Maillard and Quevauviller, 1956; Kaars Sijpesteijn and Janssen, 1959) and inhibit the growth of plant nematodes (Cave, 1958). This report is concerned with the *in vitro* and *in vivo* trichomonacidal properties of these and related compounds.

Experimental

Materials

Trichomonas vaginalis R and *Trichomonas foetus* MSC, previously described (Michaels and Strube, 1961), were used for *in vitro* assays and *in vivo* assays in mice. The C.I. strain of *T. vaginalis* was isolated by us during the course of this work. All strains were maintained in fluid thioglycollate medium with 5 per cent horse-serum (FTM) (Michaels and Strube, 1961).

In vitro *Assays*

The amount of a compound producing growth stasis in 90 per cent of organisms was determined by a two-fold dilution series of the compound involved. Solutions or finely divided suspensions of the compounds in 0·5 ml. of saline (0·9 per cent NaCl, w/v) were added to 4·5 ml. of FTM adjusted to contain an initial population of 10,000 cells/ml.

after this dilution. The highest amount of any chemical tested was 200 μg./ml.

After 22 hr. incubation at 37°, cell populations were determined by direct counts in a Neubauer chamber. The percentages of inhibition were calculated for each level of inhibitor by comparison with untreated controls. The 90 per cent end-points were read from semi-logarithmic plots of these data (Michaels and Strube, 1961).

In vivo *Assays*

Groups of 5 adult, male, albino mice (CF 1 strain) were infected subcutaneously with 200,000, 24 hr. cells in a volume of 0·2 ml. Compounds for treatment of these infections were homogenised in a sterile solution of 5 per cent gelatin. Beginning with the Tolerated Dose, which has been defined as the maximum amount of a drug in mg./kg./day which can be administered for six consecutive days and still allow weight gains comparable to those of the control animals (Michaels and Strube, 1961), drugs were given in serial two-fold dilutions either orally or intraperitoneally. The first treatment was given immediately after infection, and continued once daily for 5 days. Controls were dosed with the suspending medium.

TABLE I

In vitro ACTIVITY OF DERIVATIVES OF DITHIOCARBAMIC ACID AND BIS(THIOCARBAMOYL) SULPHIDE AND DISULPHIDE AGAINST *Trichomonas vaginalis* AND *Trichomonas foetus*

Chemical	Concentration in μg./ml. producing 90 per cent growth inhibition of	
	T. vaginalis	*T. foetus*
4-Morpholinecarbodithioic acid, sodium salt	*	0·31
1-Pyrrolidinecarbodithioic acid, sodium salt	0·64	0·45
1-Piperidinecarbodithioic acid, piperidine salt	*	1·20
Sodium dimethyldithiocarbamate	0·065	0·074
Sodium diethyldithiocarbamate	0·17	0·17
Bis(morpholinothiocarbonyl) disulphide	0·70	0·75
Bis(dimethylthiocarbamoyl) sulphide	0·37	0·31
Bis(dimethylthiocarbamoyl) disulphide	0·36	0·52
Bis(diethylthiocarbamoyl) disulphide	0·72	1·50
Bis(1-piperidinothiocarbonyl) disulphide	0·26	Not tested

* See text for explanation since there was more than one 90 per cent end point for this chemical.

Animals were killed 7 days after infection. The presence of trichomonads in the subcutaneous lesions resulting from injection was verified by microscopic examination of an eosin-stained wet smear. The 50 per cent curative dose (CD 50) was calculated by the method of Reed and Muench (1938). The relative activities of the compounds were obtained by comparing their therapeutic quotients which has been defined as the tolerated dose divided by the CD 50 (Michaels and Strube, 1961).

Compounds were also assayed in spayed, female, albino rats of the Long-Evans strain infected intravaginally with the C.I. strain of *T. vaginalis* (Cavier and Mossion, 1956; Michaels, Peterson and Stahl, 1962). The method of treatment was the same as used for mice except

that the first treatment was not given until at least 1 month after infection. Therapeutic efficacy was evaluated by the presence or absence of the organism in vaginal smears which were made daily. Animals which became negative during the course of treatment and remained so for two additional weeks were considered cured.

Results

Many derivatives of dithiocarbamic acid and of bis(thiocarbamoyl) disulphide were tested which were inactive *in vitro* by the minimal criterion established for this test, i.e., 90 per cent growth inhibition at 200 μg./ml. Sodium dimethyldithiocarbamate was the most active substance tested. It was 90 per cent growth inhibitory to *T. vaginalis* at 0·065 μg./ml. and to *T. foetus* at 0·074 μg./ml. It was the only dithiocarbamate that was distinctly more active than its oxidation products (Table I). All other compounds were also very active against both species of trichomonads and the range of activity was small (0·17–1·5 μg./ml.).

TABLE II

THE *in vitro* DOSE-RESPONSE CURVE OF *Trichomonas vaginalis* TO 4-MORPHOLINECARBODITHIOIC ACID, SODIUM SALT AND 1-PIPERIDINECARBODITHIOIC ACID, PIPERIDINE SALT

Chemical concentration, μg./ml.	Percentage of growth inhibition produced by	
	4-Morpholine-carbodithioic acid, sodium salt	1-Piperidine-carbodithioic acid, piperidine salt
200·0	100·0	97·6
100·0	97·0	98·8
50·0	91·4	99·5
25·0	88·0	98·3
12·5	93·6	73·7
6·2	85·0	89·8
3·1	85·0	85·5
1·6	89·7	91·5
0·8	98·8	75·7
0·4	43·5	58·1
0·2	0·0	0·0

The dose response of *T. vaginalis* to sodium 4-morpholinecarbodithioate and piperidine 1-piperidinecarbodithioate was unusual (Table II). Over the range 1·6–50 μg./ml., increasing the concentration of sodium 4-morpholinecarbodithioate did not necessarily increase the amount of growth inhibition, or increased it only slightly. Thus four 90 per cent end-points could be calculated at 0·71, 1·6, 7·4 and 37·4 μg./ml. A similar phenomenon was found for piperidine 1-piperidinecarbodithioate over a range of 3·1–25·0 μg./ml. Three, 90 per cent end-points could be calculated, at 1·3, 6·2 and 19·8 μg./ml.

In vivo activity is shown in Tables III and IV. There was no direct correlation of *in vivo* and *in vitro* activity. The compounds which were most active *in vitro*, sodium dimethyldithiocarbamate, sodium diethyldithiocarbamate and bis(1-piperidinothiocarbonyl) disulphide, were not active *in vivo* against *T. vaginalis* and were not tested against *T. foetus*. Piperidine-1-piperidinecarbodithioate and bis(diethylthiocarbamoyl) disulphide were active against *T. vaginalis* and not *T. foetus*. Bis(dimethylthiocarbamoyl) sulphide was much more active against *T. vaginalis* than

T. foetus. With these exceptions, *in vivo* activity was the same against both species of trichomonads. On a weight basis, all the derivatives of bisdithiocarbamic acid were equally active against *T. vaginalis* (average CD 50 about 4 mg./kg., i.p.) except bis(diethylthiocarbamoyl) disulphide which had a CD 50 of 73·7 mg./kg., intraperitoneally. There was a

TABLE III

In vivo EFFICACY OF DERIVATIVES OF DITHIOCARBAMIC ACID AND OF BIS(THIOCARBAMOYL) SULPHIDE AND DISULPHIDE ON EXPERIMENTAL INFECTIONS WITH *Trichomonas vaginalis* IN MICE

Chemical name	Tolerated dose mg./kg.		CD50* in mg./kg./day		Therapeutic quotient**	
	I.P.	Oral	I.P.	Oral	I.P.	Oral
4-Morpholinecarbodithioic acid	100	400	3·9	17·4	25	23
1-Pyrrolidinecarbodithioic acid..	800	800	8·0	20·4	100	39
1-Piperidine carbodithioic acid, piperidine salt	100	100	16·2	141	6·2	0·7
Bis(morpholinothiocarbonyl) disulphide ..	25	200	3·7	9·8	6·8	20·4
Bis(dimethylthiocarbamoyl) sulphide	100	100–200	4·5	8·8	22	11
Bis(dimethylthiocarbamoyl) disulphide ..	12·5–25	+ 200	4·5	15·6	2·8	12·8
Bis(diethylthiocarbamoyl) disulphide	100	400	73·7	200	1·4	2·0

* Curative dose 50 ** Tolerated dose/CD50

four-fold difference in the range of the CD 50 of derivatives of dithiocarbamic acid (3·9–16·2 mg./kg., i.p.), using *T. vaginalis* as the test organism. Using the criterion of the therapeutic quotient, sodium 4-morpholinecarbodithioate and sodium 1-pyrrolidinecarbodithioate were distinctly more active than any of the other compounds tested against *T. vaginalis*. The former compound also was the most active against *T. foetus*.

TABLE IV

In vivo EFFICACY OF DERIVATIVES OF DITHIOCARBAMIC ACID AND OF BIS(THIOCARBAMOYL) SULPHIDE AND DISULPHIDE ON EXPERIMENTAL INFECTIONS WITH *Trichomonas foetus* IN MICE

Chemical name	Tolerated dose mg./kg.		CD50* in mg./kg./day		Therapeutic quotient**	
	I.P.	Oral	I.P.	Oral	I.P.	Oral
4-Morpholinecarbodithioic acid	100	400	9·3	17·4	10·8	23
1-Pyrrolidinecarbodithioic acid..	800	800	10·4	inactive	76·8	
1-Piperidine carbodithioic acid, piperidine salt	100	100	inactive	not tested		
Bis(morpholinothiocarbonyl)disulphide ..	25	200	6	6·2	4·2	32·2
Bis(dimethylthiocarbamoyl) sulphide	100	100–200	37	67·3	2·7	1·5
Bis(dimethylthiocarbamoyl) disulphide ..	12·5–25	200	8·8	43	1·4	4·6
Bis(diethylthiocarbamoyl) disulphide ..	100	400	inactive	not tested		

* Curative dose 50 ** Tolerated dose/CD50

The *in vivo* dose-response curves of *T. vaginalis* and *T. foetus* to bis-(dimethylthiocarbamoyl) sulphide (Table V) and of *T. vaginalis* to sodium 1-pyrrolidinecarbodithioate (Table VI) were not linear throughout. For comparison, the response of *T. foetus* to sodium 1-pyrrolidinecarbodithioate is also shown in Table VI. It will be recalled that *T.*

foetus was unaffected by this compound when the oral route of treament was used.

Because of the unusual nature of the response to these compounds, the data have been documented as completely as possible. This type of data has never been found with 22 other compounds active systemically in mice against *T. vaginalis* which have been assayed repeatedly by this laboratory. Because of this non-linear response, sodium 1-pyrrolidine-carbodithioate was not assayed in rats.

TABLE V

THE *in vivo* DOSE-RESPONSE CURVES OF *Trichomonas vaginalis* AND *Trichomonas foetus* TO BIS(DIMETHYLTHIOCARBAMOYL) SULPHIDE IN MICE

Dose mg./kg./day	*T. vaginalis*			*T. foetus*	
	i.p.[1]	i.p.	i.p.	i.p.	oral[2]
400·0				0	5
200·0	2[3]	1		1	0
100·0	2	5	1	1	4
50·0	5	5	5	4	2
25·0	5	5	5	3	3
12·5	5	5	5	4	1
6·2	5	5	4	2	0
3·1	0	0	0	2	
1·6	0		0	1	

1. Intraperitoneal route of treatment.
2. Oral route of treatment.
3. Number of animals cured out of a total of 5 treated.

TABLE VI

THE *in vivo* DOSE-RESPONSE CURVES OF *Trichomonas vaginalis* AND *Trichomonas foetus* TO 1-PYRROLIDINECARBODITHIOIC ACID, SODIUM SALT IN MICE

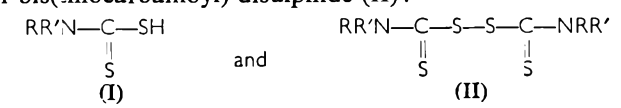
Dose mg./kg./day	*T. vaginalis*		*T. foetus*	
	i.p.[1]	oral[2]	i.p.	oral
800·0	0[3]	1		
400·0			5	
200·0	1	3		0
100·0	3		5	0
50·0	4	5	0	
25·0	5		5	0
12·5	5	1		0
6·2	1		1	0
3·1	0	0		

1. Intraperitoneal route of treatment.
2. Oral route of treatment.
3. Number of animals cured out of a total of 5 treated.

Rats infected intravaginally were treated orally with 12·5–200 mg./kg. (tolerated dose in this species) of sodium 4-morpholinecarbodithioate. The results were negative. As a positive drug control, 2-hydroxy-5-nitro-pyridine was used (Michaels and Strube, 1961). The CD 50 was 326 mg./kg.

DISCUSSION

The compounds are *N*-substituted derivatives of dithiocarbamic acid (I) and of bis(thiocarbamoyl) disulphide (II):



(I) (II)

That RR'N·CS·S· was the active structure was concluded from the *in vitro* inactivity of *NN*-dimethylthioformamide, *NN*-diethylthioacetamide, thiourea, and rubeanic acid (dithio-oxamide). When RR' was dimethyl or diethyl, the compounds were active; when RR' was dibutyl the compounds were inactive *in vitro*. The morpholino-, pyrrolidino- and piperidino-derivates of dithiocarbamic acid were active. Both the morpholino- and piperidino-derivatives were active in the disulphide series. The pyrrolidino-derivative was not tested.

The *in vitro* order of activity of substituted dithiocarbamates was Me>Et>pyrrolidino>morpholino>piperidino. This is different from the order of activity of these substances as bacteriostatic agents which was morpholino>Me>piperidino (Chabrier, Maillard and Quevauviller, 1956).

It has been found (Kaars Sipjesteijn and Janssen, 1958; 1959) that sodium dimethyldithiocarbamate forms a 1:1 complex with copper at low concentrations, at higher concentrations the 1:2 complex is formed and at still higher concentrations the free ion is present in the medium. The 1:1 complex is relatively soluble, but the 1:2 complex is relatively insoluble. Although all three molecular species are toxic to moulds and bacteria, there is a species variation in sensitivity to the three forms. Since the 1:2 complex is so insoluble, an organism must be extremly sensitive to it to be affected by it. If it is not, there will be growth over the range in which the 1:2 complex is formed. This had been called a zone of inversion growth (Kaars Sijpesteijn, Janssen and van der Kerk, 1957). The response of trichomonads to most of the derivatives of dithiocarbamic acid and of bis(thiocarbamoyl) disulphide was like the response of *Glomerella congulata* to sodium dimethyldithiocarbamate (Kaars Sijpesteijn and Janssen, 1959), i.e., all the organisms were very sensitive at low concentrations of the compounds and there was no zone of inversion growth.

The response of *T. vaginalis* to sodium 4-morpholinecarbodithioate and piperidine 1-piperidinecarbodithioate does not fit exactly any previously described dose-response relationship to these kinds of compounds. Rather than a zone of inversion growth a zone of non-response was found. This zone of non-response was probably due to the formation of an insoluble 1:2 copper complex with the result that the amount of compound in solution increased very slowly over this range.

A real zone of inversion growth was found *in vivo* but to compounds whose *in vitro* response was linear. Whether this was due to chelation with a different metal *in vivo* than *in vitro*, or to an entirely different mechanism, is not known.

Dithiocarbamates and bis(thiocarbamoyl) disulphides were as active *in vitro* against *T. vaginalis* as acinitriazole(2-acetamido-5-nitrothiazole) and 2-amino-5-nitropyridine, more active than 2-amino-5-nitropyrimidine, and less active than the di(4-methyl-3-thiosemicarbazone) of pyruvaldehyde (Michaels and Strube, 1961; Michaels, Peterson and Stahl, 1962). The *in vivo* activity against experimental infections with *T. vaginalis* in mice was equal to or greater than any of the compounds mentioned

excepting the di(4-methyl-3-thiosemicarbazone) of pyruvaldehyde. However, it is not recommended that these compounds be tried clinically since they did not cure vaginal infections in rats and they are extremely toxic (Dubois, Raymund and Hietbrink, 1961).

REFERENCES

Cave, A. S. (1958). Australian Patent Application, 42639/58.
Cavier, R. and Mossion, X. (1956). *C.R. Acad. Sci., Paris*, **243**, 1807–1809.
Chabrier, D., Maillard, G. and Quevauviller, A. (1956), *Ann. pharm. franç.*, **14**, 720–728.
Dubois, K. P., Raymund, A. B. and Hietbrink, B. E. (1961). *Toxicol. Appl. Pharmacol.*, **3**, 236–255.
Kaars Sijpesteijn, N. and Janssen, M. J. (1958). *Nature, Lond.*, **182**, 1313–1314.
Kaars Sijpesteijn, N. and Janssen, M. J. (1959). *Antonie van Leeuwenhoek*, **25**, 422–438.
Kaars Sijpesteijn, N., Janssen, M. J. and van der Kerk, G. J. M. (1957). *Biochim. et Biophys. Acta*, **23**, 550–557.
Michaels, R. M., Peterson, L. J. and Stahl, G. L. (1962). *J. Parasitol.*, in press.
Michaels, R. M. and Strube, R. E. (1961). *J. Pharm. Pharmacol.*, **13**, 601–610.
Reed, L. J. and Muench, H. (1938). *Amer. J. Hyg.*, **27**, 493–497.

SOME *IN VITRO* TESTS ON SODIUM GLUCONATODIHYDROXOALUMINATE III: A SOLUBLE BUFFER ANTACID

BY F. GROSSMITH

From Beecham Research Laboratories Ltd., Product Research Division, Brentford, Middlesex

Received July 30, 1962

The properties of a new buffer antacid, the active substance of which is a 40 per cent w/v aqueous solution of a complex considered to be sodium gluconatodihydroxoaluminate III, have been compared with those of some existing antacid preparations. The unit dose (4 ml.) of this preparation has high acid neutralising capacity, speed of action and buffering power.

VARIOUS compositions have been used as liquid buffer antacids, for example, aluminium hydroxide gel preparations, aluminium phosphate gel and magnesium trisilicate gel, alone or as mixtures. Such preparations are suspensions of insoluble substances generally of unattractive flavour and texture. Aluminium hydroxide gel is frequently astringent to the oral mucosa and has a flocculent texture.

A soluble buffer antacid of acceptable flavour would represent an advance on the existing preparations. The minimum requirements include palatability, and solubility associated with a good acid consuming capacity to permit an effective dose in a reasonable bulk desirably buffering in the physiological pH range of 5 to 3. Hitherto, no compound has been available which met these requirements, but a new preparation based on a complex derived from sodium aluminate, aluminium hydroxide and glucono delta lactone, appears to do so. The complex has a composition corresponding to sodium gluconatodihydroxoaluminate III. The formula is considered to be

$$Na[Al(OH)_2(C_6H_{10}O_7)]$$

Molecular weight = 278·16

Content of Al 9·70 per cent (dry basis)

Content of Na 8·27 per cent (dry basis).

Properties

The complex is an odourless, off-white, non-crystalline solid of bland flavour. Its solubility in water at 25° is over 80 per cent w/v. It is insoluble in dehydrated ethanol, ether and chloroform. A 30 per cent w/v solution has a pH of 8·5. Acid neutralising capacity: when 1 g. is digested with 10 ml. N hydrochloric acid on a water bath for 1 hr., the pH is raised to 3.

Antacid Properties

The desirable characteristics of an antacid may be defined as: (1) high acid neutralising capacity; (2) rapid action; (3) not alkaline, even in conditions of overdosage; (4) without significant systemic effects; (5) not flocculent or astringent to the oral mucosa; (6) neither constipating nor

excessively laxative; (7) pepsin inhibiting, but not inactivated by pepsin or peptones; (8) palatability.

To investigate points 1, 2 and 3, four tests were selected and the new complex was compared with five liquid suspension antacids and two antacid tablets of the quick-acting buffer type. Table I lists the preparations and the active ingredients tested. With one exception the comparisons were made with a unit dose of the preparation (4 ml. or one tablet). This was regarded as appropriate since, if the unit dose has a low content of active substance, there are other limiting factors such as viscosity, the thixotropic character or the stability of a liquid suspension, or the size of a tablet.

TABLE I

COMPOSITION OF PREPARATIONS TESTED

Preparation	Content of active agent
40 per cent w/v aqueous solution of sodium gluconato-dihydroxoaluminate III	4 ml. contains 1·6 g. of the complex
Mixture of magnesium carbonate B.P.C.	½ fluid oz. contains: light magnesium carbonate 0·343 g. sodium bicarbonate 0·515 g.
Aluminium hydroxide gel B.P.	4 ml. contains: aluminium hydroxide 0·224 g.
Aluminium hydroxide/magnesium trisilicate gel	4 ml. contains: aluminium hydroxide 0·25 g. magnesium trisilicate 0·50 g.
Magnesium hydroxide gel	4 ml. contains: magnesium hydroxide 0·357 g.
Aluminium phosphate gel	4 ml. contains: aluminium phosphate 0·327 g.
Aluminium hydroxide-magnesium carbonate co-dried gel (Tablet)	Each tablet contains: co-dried gel 0·375 g.
Sodium polyhydroxy-aluminium monocarbonate hexitol complex (Tablet)	Each tablet contains: complex 0·360 g.

An exception to the unit dose basis was made for the B.P.C. mixture of magnesium carbonate, where half the minimum B.P.C. dose of ½ fluid oz. was taken as this was a more appropriate quantity for the tests.

The tabletted products were powdered and prepared as a slurry.

To investigate the inhibition of antacid activity by pepsin and peptone, the test complex was compared with the aluminium hydroxide gel B.P., the sodium polyhydroxyaluminium monocarbonate hexitol complex tablets and the aluminium hydroxide-magnesium carbonate co-dried gel tablets.

EXPERIMENTAL

Antacid Activity Test

This is the test of Gore, Martin and Taylor (1953) modified to use the unit dose, or other quantity, as defined. The dose of antacid is introduced into a 200 ml. pool containing 3 m-equiv. of hydrochloric acid giving the solution a normality of 0·015N. The pool of acid is contained in a beaker

holding a saturated calomel and wide range glass electrode assembly and stirrer connected to a direct reading Cambridge pH meter and recorder. The pH is recorded for 30 min. and further hydrochloric acid is then injected by a motor and worm driven syringe at the rate of 6 m-equiv./hr. to an end-point of pH 3, the rate of change of pH with time being automatically recorded throughout. The test gives information about the speed of neutralisation, the pH reaction to over-dosing, acid neutralising capacity and buffering capacity.

Acid-consuming Capacity Test

This is a modification of the U.S.P. test (1960). It measures the amount of 0·1N hydrochloric acid neutralised or absorbed by the antacid when it is digested with excess of acid at 37° for 1 hr. The excess is titrated with sodium hydroxide to pH 3.

Speed of Neutralisation Test

In this test the unit dose of antacid is introduced into 200 ml. pools at 37° containing 0·1N hydrochloric acid in progressively larger amounts, and the time necessary for pH 3 to be attained in each of the pools is recorded. When the acidity of the pool exceeds the capacity of a unit dose of antacid, the pH never attains 3; then, using a pool not more than 5 ml. 0·1N hydrochloric acid in excess of that last giving pH 3, the value of the pH after 40 min. is determined and recorded as the final observation.

Speed and Buffering Capacity Test

The amount of N hydrochloric acid necessary to give a "weak" and a "strong" 200 ml. pool at 37°, of a strength such that when a unit dose of antacid was introduced the pH reached after 5 min. was at least 3·0 and after 30 min. was not greater than 5·0, was ascertained.

Inhibition of Antacid Activity by Pepsin and Peptone

The B.P. neutralising test was employed in the first instance as specified in the B.P. (1958) and then repeated substituting the artificial gastric juice described by Brindle (1953) for the hydrochloric acid. With aluminium hydroxide gel B.P. the amount specified in the B.P. test was used (5 g. ≡ 4·8 ml.), while the unit dose of 4 ml. of test complex and two tablets of sodium polyhydroxyaluminium monocarbonate hexitol complex and aluminium hydroxide-magnesium carbonate co-dried gel were taken.

Results

Antacid Activity Test

The results obtained in the antacid activity test are as shown graphically in Fig. 1.

With the complex the pH rose almost instantly to a value of 4, and reached its maximum value of pH about 5 after 10 min. and remained

at about 5 for the next 20 min. during which no acid was injected. This indicated that the pH reaction to over-dosing *in vivo* might be expected not to rise above pH 5. When further acid was injected, the pH did not fall below 3 until approximately a total of 15 m-equiv. of hydrochloric acid had been injected.

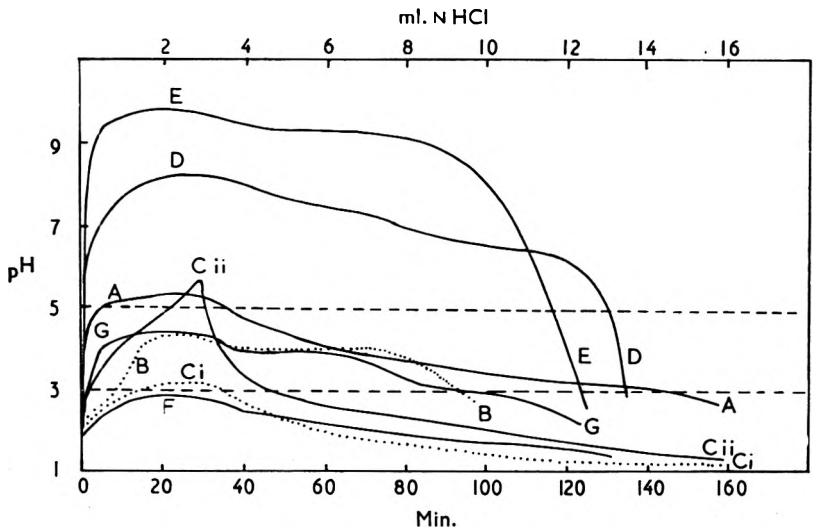


FIG. 1. Antacid activity test (Method of Gore, Martin and Taylor, 1953).

A, 40 per cent w/v aqueous solution of sodium gluconatodihydroxoaluminate III, 4 ml. B, Aluminium hydroxide gel B.P., 4 ml. C, Aluminium hydroxide/magnesium trisilicate gel, 4 ml. (i) Sample 1. (ii) Sample 2. D, Mixture of magnesium carbonate B.P.C., $\frac{1}{4}$ fl. oz. E, Magnesium hydroxide gel, 4 ml. F, Aluminium phosphate gel, 4 ml. G, Aluminium hydroxide/magnesium carbonate co-dried gel, 1 tablet.

The other antacids tested were less rapid in their action and gave curves resembling those previously published for similar types of tests, for example, Beekman (1960). It is also noteworthy that some of the preparations (magnesium hydroxide gel and mixture of magnesium carbonate B.P.C.) took the pH into the acid rebound range and would not show up any better in this test if a different quantity of active substance were taken. Other preparations (aluminium hydroxide/magnesium trisilicate gel and aluminium phosphate gel) could not be expected to give a significantly improved performance unless a multi-dose quantity was taken. With the aluminium hydroxide/magnesium trisilicate gel a second sample of the preparation from the same source was found to be markedly more reactive, although the acid consuming capacities were the same for the two samples.

The aluminium hydroxide/magnesium carbonate co-dried gel tablets had a good neutralising capacity within the pH range 3–5 and had a superior speed of action to the aluminium hydroxide gel.

Acid Consuming Capacity Test

The results obtained from the acid consuming capacity test are shown in Table II. The unit dose of the test substance can be seen to have neutralised more acid than unit doses of the other preparations examined.

TABLE II

ACID CONSUMING CAPACITY TEST (U.S.P. MODIFIED)

Product	Quantity	ml. 0·1N HCl neutralised
Sodium gluconatodihydroxoaluminate III in 40 per cent w/v aqueous solution	4 ml.	160 ml.
Magnesium hydroxide gel	4 ml.	125 ml.
Aluminium hydroxide gel B.P.	4 ml.	115 ml.
Aluminium hydroxide-magnesium carbonate co-dried gel	1 tablet	103 ml.
Sodium polyhydroxyaluminium monocarbonate hexitol complex	1 tablet	88 ml.
Aluminium hydroxide/magnesium trisilicate gel	4 ml.	79 ml.
Aluminium phosphate gel	4 ml.	18 ml.

Speed of Neutralisation Test

The results obtained from the speed of neutralisation test are depicted in Fig. 2.

With most antacids the speed of neutralising acid in this test varies markedly with the acidity of the pool to be neutralised. This is not true of the test complex and also of magnesium hydroxide gel which responded almost instantaneously, irrespective of the acidity of the pool up to about 12 m-equiv. of hydrochloric acid, which is about 75 per cent of the total neutralising capacity of the dose of the complex and over 90 per cent of the dose of the gel.

Speed and Buffering Capacity Test

The results of the speed and buffering capacity test are given in Table III.

TABLE III

SPEED AND BUFFERING CAPACITY

Product	Quantity	ml. 1·0N HCl buffered in the pH range 3–5 between 5 and 30 min.	
		Minimum	Maximum
Sodium gluconatodihydroxoaluminate III in 40 per cent w/v aqueous solution	4 ml.	3·5	12·0
Aluminium hydroxide gel B.P.	4 ml.	0·4	8·7
Sodium polyhydroxyaluminium monocarbonate hexitol complex	1 tablet	0·7	7·0
Aluminium hydroxide-magnesium carbonate co-dried gel	1 tablet	1·1	8·1
Magnesium hydroxide gel	4 ml.	11·6	11·7
Mixture of magnesium carbonate B.P.C.	½ fluid oz.	6·8	7·3
Aluminium hydroxide/magnesium trisilicate gel	4 ml.	4·7	5·0
Aluminium phosphate gel	4 ml.	0·1	0·7

For rapid buffering, the test complex covers a wider range of acidities (8·5 ml. 1·0N HCl) than the other antacids. Aluminium hydroxide gel B.P., sodium polyhydroxyaluminium monocarbonate hexitol complex and

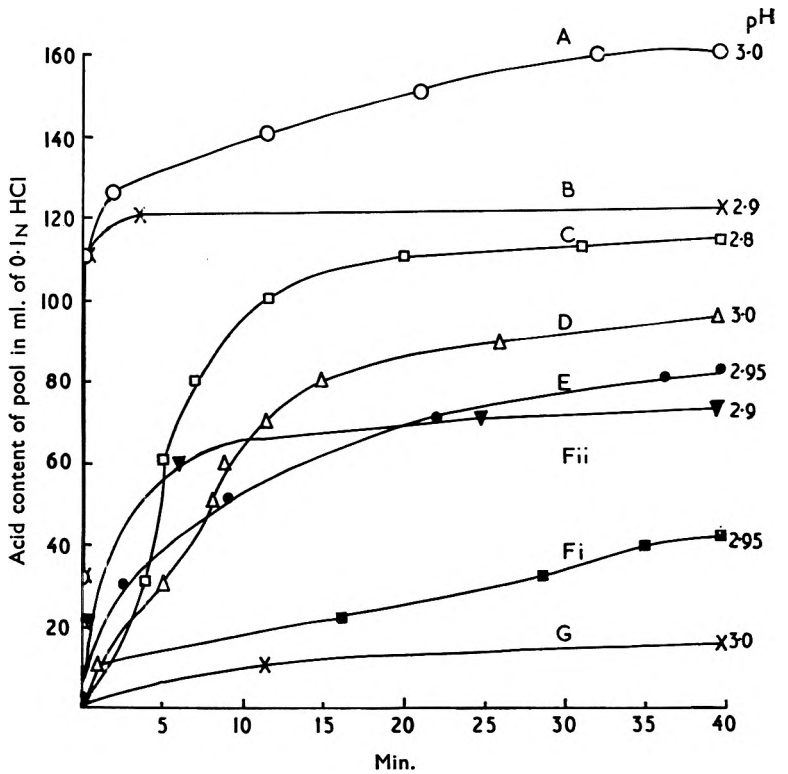


FIG. 2. Speed of neutralisation test. Each experimental observation was obtained by adding a dose of antacid to a 200 ml. acid pool at 37°. The points mark the acidity of the pools and the times to reach pH 3, except for the 40 min. points where the pH attained is shown.

A	40 per cent w/v aqueous solution of sodium gluconato-dihydroxoaluminate III	4 ml.
B	Magnesium hydroxide gel	4 ml.
C	Aluminium hydroxide gel B.P.	4 ml.
D	Aluminium hydroxide/magnesium carbonate co-dried gel	one tablet
E	Sodium polyhydroxyaluminium monocarbonate hexitol complex	one tablet
F	Aluminium hydroxide/magnesium trisilicate gel	4 ml.
	(i) Sample 1	
	(ii) Sample 2	
G	Aluminium phosphate gel	4 ml.

the aluminium hydroxide-magnesium carbonate co-dried gel nearly equal this buffering range, for while their maximum capacities are lower, they are able to deal with smaller amounts of acid without exceeding pH 5·0. No disadvantage is considered to be associated with the test complex minimum of 3·5 ml. of N hydrochloric acid, however, as this ratio of antacid to acid corresponds to a considerable degree of over-dosing *in vivo*.

The remaining antacids examined are shown in this test to have limited buffering capacities.

Inhibition of Antacid Activity by Pepsin and Peptone

The results of the tests for the inhibition of antacid activity by pepsin and peptone for the three preparations tested are given in Table IV and graphically for two of the antacids in Fig. 3.

TABLE IV

INHIBITION OF ANTACID ACTIVITY BY PEPSIN AND PEPTONE

B.P. Neutralising Capacity Test (*a*) with HCl, and (*b*) with artificial gastric juice (Brindle, 1953)

	Initial pH	pH after 10 min.	pH after 15 min.	pH after 20 min.	Back titration 0·1N NaOH
B.P. Standard—not less than	—	1·8	2·3	3·0	Not more than 50 ml.
Sodium gluconatodihydroxoaluminate III in 40 per cent w/v aqueous solution (4 ml.)—					
0·05N HCl	1·35	3·5	3·55	3·6	23 ml.
Gastric juice	1·45	3·4	3·45	3·5	28 ml.
Aluminium hydroxide gel B.P. (5 g. = 4·8 ml.)—					
0·05N HCl	1·35	3·65	3·70	3·73	38·6 ml.
Gastric juice	1·45	2·44	2·78	2·98	44·8 ml.
Sodium polyhydroxyaluminium monocarbonate hexitol complex (2 tablets)—					
0·05N HCl	1·35	3·85	3·9	3·95	not
Gastric juice	1·45	2·6	2·78	2·9	applicable
Aluminium hydroxide-magnesium carbonate co-dried gel (2 tablets)—					
0·05N HCl	1·35	4·0	4·05	4·05	not
Gastric juice	1·45	3·4	3·55	3·65	applicable

The inhibiting effect on the activity of the sodium gluconatodihydroxoaluminate III complex is insignificant, while it is appreciable for the aluminium hydroxide-magnesium carbonate co-dried gel and considerable for the aluminium hydroxide gel B.P. and for the sodium polyhydroxyaluminium monocarbonate hexitol complex. As the extent of loss of activity is similar in both instances only one is compared with the test complex in Fig. 3.

DISCUSSION

The tests described suggest that sodium gluconatodihydroxoaluminate III would be an effective soluble buffer antacid compound. The preparation compares favourably with similar doses of other antacids for acid consuming capacity, speed of neutralisation and buffering power. The tests also suggest that acid rebound would be unlikely *in vivo*.

Unlike aluminium hydroxide gel B.P. and some other preparations containing it, the antacid activity of sodium gluconatodihydroxoaluminate III is not significantly depressed by pepsin and peptone. Newey (1962) has drawn attention to the fact that the effect of peptone on antacid activity is more significant than that of pepsin. Peptones are among the intermediate soluble breakdown products of proteins, the disruption being accelerated by enzyme action. Pepsin and peptones may therefore be expected to be present in the stomach after meals and it is relevant to consider their interaction with antacids. It is obviously desirable to

avoid inactivating the antacid: in addition, reduction of the proteolytic activity of pepsin to safeguard against the auto-digestion of impaired mucosal tissue, without complete inhibition which would stop normal digestion, is desirable. The proteolytic action of pepsin diminishes with increasing pH, losing about 80 per cent of its power at pH 4 (Douthwaite, 1958).

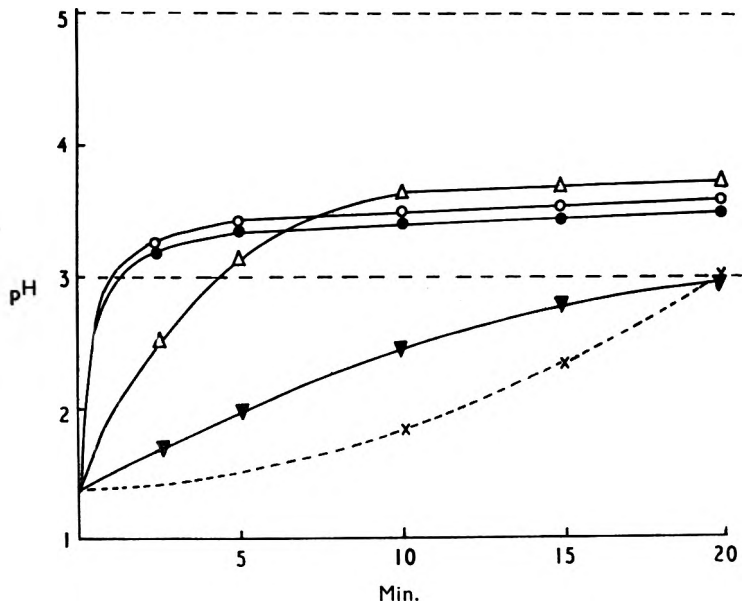


FIG. 3. Inhibition of antacid activity by pepsin and peptone. The B.P. neutralising capacity test for aluminium hydroxide gel was carried out and then repeated using artificial gastric juice containing 0·15 per cent each NaCl, pepsin and peptone in 0·05N HCl.

C—O 4 ml. sodium gluconatodihydroxoaluminate III (40 per cent w/v aqueous solution) in HCl.
●—● 4 ml. sodium gluconatodihydroxoaluminate III in artificial gastric juice.
△—△ 5 g. (4·8 ml.) of aluminium hydroxide gel B.P. in HCl.
▼—▼ 5 g. (4·8 ml.) of aluminium hydroxide gel B.P. in artificial gastric juice.
X--X B.P. test minimum pH values.

Sodium gluconatodihydroxoaluminate III, therefore, would be expected to permit a reduced amount of peptic digestion to proceed except under conditions of over-dosage. It is often stated (for example, Almy and Steinberg, 1958–59) that aluminium hydroxide possesses a specific pepsin inhibiting action independent of pH. This aspect has not been investigated in the present study but such an effect, and also the reduction of the antacid action of aluminium hydroxide by peptone would be consistent with a precipitating action of aluminium hydroxide on proteins.

We have found that sodium gluconatodihydroxoaluminate III does not precipitate proteins, for example, when it is added to a solution of casein.

There is probably a connection between this latter fact and the finding that the complex is not astringent to the oral mucosa.

Histological and biochemical studies, and chronic toxicity tests on dogs have so far shown (Mr. D. M. Brown, personal communication) the complex to be non-toxic.

Acknowledgements. Acknowledgement is made to Messrs. E. E. Berry, J. Davis and R. G. Englefield who did most of the experimental work described and contributed to the interpretation of the results.

REFERENCES

Almy, T. P. and Steinberg, H. (1958–1959). *Drugs of Choice*, p. 377.
Beekman, S. M. (1960). *J. Amer. Pharm. Ass., Sci. Ed.*, **49**, 191–200.
Brindle, H. (1953). *J. Pharm. Pharmacol.*, **5**, 692–702.
British Pharmacopoeia (1958). 31.
Douthwaite, A. H. (1958). *Proc. Roy. Soc. Med.*, **51**, 1066.
Gore, D. N., Martin, B. K., and Taylor, M. P. (1953). *J. Pharm. Pharmacol.*, **5**, 686–691.
Newey, A. E. (1962). *Pharm. J.*, **188**, 115.
Pharmacopoeia of the United States XVI (1960), 32, 391.

THE ALIMENTARY ABSORPTION OF SOME ENTERIC-COATED SODIUM AND POTASSIUM CHLORIDE TABLETS

By Victor Wynn and John Landon

From the Surgical Unit, St. Mary's Hospital, London

Received August 21, 1962

The alimentary absorption of sodium chloride and potassium chloride tablets covered with a new enteric coating, has shown their absorption to be satisfactory. Of three other brands, one has been found to be only poorly absorbed, producing diarrhoea in some subjects; a second brand was poorly absorbed in 1 out of 5 patients, while the third proved moderately satisfactory. Samples of all the brands of enteric-coated tablets passed the *in vitro* tests for disintegration specified by the British Pharmacopoeia (1958, Appendix 21B).

An enteric coating is useful when the material to be absorbed may cause gastric irritation. Sodium, potassium and ammonium chloride are often prescribed as enteric-coated tablets, as are salicylates and some glucocorticoids. Enteric-coated tablets should comply with the test for disintegration described in Appendix 21B of the British Pharmacopoeia, 1958. However, tablets which conform to this specification are sometimes excreted intact, or only partially digested, in the stools. Pirnie and Staffurth (1961) drew attention to this problem and suggested a revision of the British Pharmacopoeia specification.

We have examined in man the absorption of sodium and potassium chloride "nuseals"* enteric-coated tablets. Three other commercially available enteric-coated potassium chloride tablets have also been investigated, but less extensively.

Methods

Various enteric-coated tablets† were given to 28 patients suffering from a variety of illnesses. In only 2 patients, cases 2 and 11, was there any known bowel disorder, and in both the disorder was mild. Twenty patients were studied in the metabolic ward and they had a constant food intake. Eight patients were studied in an orthopaedic ward, their diet was kept as constant as possible.

Faeces were collected for consecutive 3-day periods, and each collection was homogenised with distilled water in a blender and analysed for sodium and potassium by flame photometry. The average daily faecal sodium and potassium for 3 or 6 days before administration of the test tablets was subtracted from the average daily excretion during the administration of the tablets to estimate the amount of administered salt

* The "nuseal" is of commercial manufacture; the coating contains the following ingredients according to the manufacturers: cellulose acetate phthalate, acacia, sucrose, gelatin, calcium sulphate and talc (hydrated magnesium silicate). The red dye is ponceaux S.X.

† The symbols A, B, C and D are used to describe the four brands of tablets. A are "nuseals." The remaining tablets will be identified on application to the authors.

TABLE I

ABSORPTION OF POTASSIUM CHLORIDE TABLETS (A)

Case	Diagnosis	Duration of therapy (days)	Faecal K before therapy, m-equiv./3 days	Mean, m-equiv./day	Faecal K during therapy, m-equiv./3 days	Mean, m-equiv./day	Loss of K supplement in faeces, per cent
1	Mainutrition K depletion	39	30	10·0	27, 21, 24, 30, 42, 30, 27, 27, 27, 24, 45, 18, 28	9·5	0
2	Mild steatorrhoea	36	42	14·0	87, 81, 108, 51, 33, 12, 51, 63, 33, 72, 21, 27	18·0	3
3	Cushing's Syndrome	21	54, 62, 48	18·0	45, 66, 39, 33, 81, 39, 63	17·0	0
4	Malnutrition K depletion	18	42, 54, 51	16·0	39, 54, 39, 39, 42, 45	14·0	0
5	Hirsutes	12	27, 31	9·5	30, 37, 15, 30	9·0	0

* Each patient received 134 m-equiv./day (10 g.).

TABLE II

ABSORPTION OF SODIUM AND POTASSIUM CHLORIDE TABLETS (A) ADMINISTERED CONCURRENTLY

Case	Diagnosis	Duration of therapy (days)	Dose of NaCl m-equiv./day (g./day)	Faecal Na				Loss of Na supplement in faeces per cent	Dose of KCl m-equiv./day (g./day)	Faecal K				Loss of K supplement in faeces per cent
				Before therapy m-equiv./3 days	Mean m-equiv./day	During therapy m-equiv./3 days	Mean m-equiv./day			Before therapy m-equiv./3 days	Mean m-equiv./day	During therapy m-equiv./3 days	Mean m-equiv./day	
6	Salt-losing pyelonephritis	36	171(10)	9, 10	3	9, 15, 9, 6, 3, 9, 6, 50, 27, 75, 48, 26	8	3	54(4)	39, 45	14	39, 93, 65, 42, 30, 45, 27, 108, 98, 90, 78, 56	19	9
7	Salt-losing pyelonephritis	30	203(12)	4, 5	2	5, 2, 28, 21, 15, 4, 6, 3, 5, 3	3	0	108(8)	9, 33	7	18, 9, 90, 78, 63, 24, 66, 24, 48, 15	16	8
8	Salt-losing pyelonephritis	9	342(20)	30	10	81, 66, 93	27	5	54(4)	36	12	54, 60, 78	21	17
9	Malnutrition	12	171(10)	9, 11	3	15, 15, 30, 25	7	2	67(5)	24, 45	12	36, 32, 23, 45	11	0

excreted. This value was then expressed as a percentage of the dose given.

In all the tests 0·5 g. tablets were used.

Results

Table I shows the results of the study of the absorption of potassium chloride tablets A given to 5 patients. The daily dose was 10 g. (20 × 0·5 g. tablets) and the longest duration of the administration was 39 days. In these 5 patients absorption of the tablets was complete.

Table II reports a study of 4 patients to whom it was necessary to give both sodium chloride and potassium chloride tablets A, each 0·5 g., in large doses. The longest duration of study was 36 days. Despite the large number of tablets taken the absorption of sodium chloride was virtually complete and the faecal loss of potassium was small. No whole tablet or recognisable fragment appeared in any of the stools.

The only symptoms noticed during the administration of these tablets occurred in Case 1 who complained of a "colicy" abdominal pain on several occasions during the first two days. The pain disappeared despite the continued administration of the tablets.

Table III shows the results from three other brands of enteric-coated potassium chloride tablets given to patients. In the patients given tablets B (Cases 10–15) absorption was fair in cases 10 and 11 and poor in the remaining four. In these 4 patients the tablets produced diarrhoea and the stools contained many whole tablets and fragments of tablets.

Five patients, 16–20, were given enteric-coated potassium chloride tablets C and these were absorbed satisfactorily, except in one patient, 16, in whom the tablets produced increased frequency of defaecation. His stools contained fragments of undigested tablets and the faecal loss of potassium increased progressively, exceeding 40 per cent of the supplementary potassium for the last 3 days of the test. Five patients, 21–25, were given enteric-coated potassium chloride tablets D. Absorption was good in two patients, 22 and 23, and fair in three, 21, 24 and 25. In two patients, 21 and 25, two whole tablets appeared in the stools during the test period.

Table IV reports the study of a cross-over experiment designed to compare the absorption of potassium chloride tablets A with tablets B. The patients were given tablets A first and then, after a suitable interval, they were given the equivalent dose of tablets B. As before, tablets A were well absorbed and tablets B were poorly absorbed.

Table V reports the results of disintegration tests made on samples of the tablets used. All the enteric-coated tablets conformed to the B.P. standards for disintegration.

Discussion

High doses of sodium chloride and potassium chloride are frequently indicated in medicine. Disintegration of tablets containing sodium or potassium chloride in the stomach may lead to gastric irritation and

TABLE III

ABSORPTION OF OTHER BRANDS OF ENTERIC-COATED POTASSIUM CHLORIDE TABLETS

Tablet	Case	Diagnosis	Duration of therapy (days)	Faecal K			Mean m-equiv./day	Loss of K supplement in faeces, per cent	Total no. of KCl tablets in faeces
				Before therapy m-equiv./3 days	Mean m-equiv./day	During therapy m-equiv./3 days			
B	10	Asthma	9	24	8	54, 84, 97	26	22	3
	11	Crohn's disease	12	27, 23	8	42, 62, 115, 45	22	17	6
81 m-equiv./day	12	Idiopathic osteoporosis	12	25, 36	10	141, 186, 116, 229	56	57	9
(6 g./day)	13	Orthopaedic	9	12	4	78, 129, 117	32	35	9
	14	Orthopaedic	9	19, 21	7	93, 87, 134	35	35	25
	15	Orthopaedic	9	31, 24	9	165, 240, 276	76	83	33
	16	Chronic bronchitis	9	40, 39	13	90, 122, 136	39	32	Many fragments
C	17	Cushing's Syndrome (iatrogenic)	9	36, 31	11	37, 34, 39	12	0	0
81 m-equiv./day	18	Idiopathic hypercalcuria	9	15	5	29, 20, 30	9	5	0
(6 g./day)	19	Anorexia nervosa	9	30, 46	13	37, 25, 46	12	0	0
	20	Chronic nephritis	9	8	3	12, 11, 11	4	0	0
D	21	Orthopaedic	9	33, 35	11	75, 63, 69	23	15	2
	22	Orthopaedic	9	27	9	0, 42, 42	9	0	0
81 m-equiv./day	23	Orthopaedic	9	62, 34	16	63, 42, 40	16	0	0
(6 g./day)	24	Orthopaedic	9	15	5	81, 27, 25	15	12	0
	25	Orthopaedic	9	12, 18	5	51, 42, 59	17	15	2

TABLE IV

CROSS-OVER EXPERIMENTS TO COMPARE ABSORPTION OF TWO BRANDS OF POTASSIUM CHLORIDE TABLETS, A AND B

Case	Diagnosis	Faecal K				Loss of supplement in faeces, per cent	Faecal K				Loss of supplement in faeces, per cent
		Before tablet A therapy m-equiv./ 3 days	Mean m-equiv./ day	During tablet A therapy m-equiv./ 3 days	Mean m-equiv./ day		Before tablet B therapy m-equiv./ 3 days	Mean m-equiv./ day	During tablet B therapy m-equiv./ 3 days	Mean m-equiv./ day	
26	Anorexia nervosa	40, 20	10	27, 19, 24	8	0	15, 25	8	81, 181, 221	54	57*
27	Anorexia nervosa	27	9	27, 72, 57	17	10	33, 35	11	108, 95, 119	36	31*
28	Obesity	65, 54	20	64, 67, 44	19	0	50, 58	18	102, 130, 162	44	32*

In each case the dose of KCl was 6 g./day (81 m-equiv.) as 0·5 g. tablets.

* Analysis of the 3 day stool from the 9th–12th day contained higher amounts of K than was to be expected from the control excretion level because of the continued excretion of unabsorbed KCl. If this additional loss is included, the corrected figures for the loss of KCl supplement becomes 63, 38 and 35 per cent, respectively.

dyspeptic symptoms. To avoid this, enteric coatings have been devised to resist disintegration in the stomach, while rapidly disintegrating in the small intestine, so allowing absorption of their contents. A risk of such coatings is that they may resist disintegration in the small intestine, the contents being wholly or partially lost in the faeces.

TABLE V

STUDY OF DISINTEGRATION OF FOUR TYPES OF ENTERIC-COATED POTASSIUM CHLORIDE TABLETS

Sample*	Results of immersion in acid-pepsin solution for 3 hr.	Results of immersion in alkaline-pancreatin solution
A	Red coating dissolved within 5 min. All 5 intact after 3 hr.	1 disintegrated in 22 min. 2 ,, ,, 22 min. 3 ,, ,, 30 min. 4 ,, ,, 32 min. 5 ,, ,, 35 min.
B_1	All 5 tablets intact at the end of 3 hr. Brown coating intact.	1 disintegrated in 20 min. 2 ,, ,, 35 min. 3 ,, ,, 40 min. 4 and 5 ,, ,, 45 min. All coating gone,, 47 min.
B_2	All 5 tablets still intact at the end of 3 hr.	1 ,, ,, 25 min. 2 ,, ,, 27 min. 3 ,, ,, 32 min. 4 ,, ,, 34 min. 5 ,, ,, 36 min. All coating gone,, 45 min.
C	Brown sugar coating dissolved within 10 min. of immersion. Tablets intact after 3 hr.	1 tablet disintegrated in 20 min. Coating cracked on all others within 23 min. 2 disintegrated in 25 min. 3 ,, ,, 30 min. 4 ,, ,, 35 min. 5 ,, ,, 40 min.
D	Sugar coating dissolved within 10 min. All 5 intact after 3 hr.	Coating split after 8 min. 1 disintegrated in 13 min. 2 ,, ,, 16 min. 3 ,, ,, 26 min. 4 ,, ,, 26 min. 5 ,, ,, 35 min. Coating still visible after 60 min.

* Five tablets were used for each test.

The tablets most intensively investigated in this study (A) have proved satisfactory for prolonged administration. Indeed, one patient (6) has been on continuous treatment with 10 g. of sodium chloride and 4 g. potassium chloride for 18 months with satisfactory results, while another (8) was treated with 20 g. of sodium chloride and 4 g. potassium chloride for 13 months with satisfactory results. She died suddenly of uraemic heart failure and at autopsy the gut was free from undissolved tablets. These two patients have demonstrated the feasibility of using large doses of enteric-coated sodium and potassium chloride tablets for prolonged periods without undesirable effects. The results of the short-term studies reported here confirm the favourable clinical impression we have formed of the use of tablets A in a variety of patients.

It was not possible to subject the other enteric-coated tablets mentioned to the same extensive test as has been applied to tablet A so that a comparison cannot be attempted. It is clear, however, that one brand of tablets used in this study is quite unsuitable for clinical use despite the

fact that it successfully passes the *in vitro* test specified by the British Pharmacopoeia.

Acknowledgments. We are indebted to Miss G. Robertson, Mr. P. Wells and Mr. G. Paulessen for making the analyses, and to Sister Gibbons and the nursing staff for their co-operation. We also wish to thank Mr. J. W. Lowe, Chief Pharmacist, St. Mary's Hospital, for doing the *in vitro* disintegration tests. Our thanks are also due to Eli Lilly & Co. Ltd. for supplying the "nuseals" used in this study, and for financial assistance.

REFERENCE

Pirnie, J. S., and Staffurth, J. S. (1961). *Lancet*, **1**, 48–49.

A NEW CATIONIC ANTIMICROBIAL AGENT, *N*-DODECYL-4-AMINOQUINALDINIUM ACETATE (LAUROLINIUM ACETATE)

By W. A. Cox and P. F. D'Arcy*

From the Research Division, Allen & Hanburys Limited, Ware, Hertfordshire

Received July 31, 1962

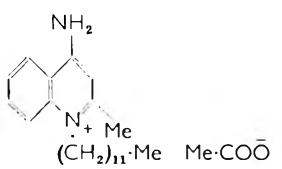
The properties of a new cationic antimicrobial agent, *N*-dodecyl-4-aminoquinaldinium acetate (laurolinium acetate, Laurodin) are described. It has bacteriostatic and bactericidal activity against Gram-positive and Gram-negative bacteria, fungi and some protozoa. Its activity is antagonised by anionic substances but some is retained in the presence of organic matter. The compound is well suited for topical use but is too toxic for parenteral use.

The antimicrobial activity of the polymethylene bis-4-aminoquinaldinium series of compounds has been studied by Babbs, Collier, Austin, Potter and Taylor (1956), Collier, Cox, Huskinson and Robinson (1959), and Cox and D'Arcy (1961). The allied *N*-alkyl derivatives of 4-aminoquinoline and 4-aminoquinaldine have antibacterial activity (Cox and D'Arcy, 1959; Caldwell, Cox, D'Arcy and Rowe, 1961). In particular, the *N*-dodecyl acetate member of the 4-aminoquinaldine series (laurolinium acetate, Laurodin†) was shown to have bacteriostatic and bactericidal activity. This has been further studied and the results are now described.

Experimental

Materials and Methods

Compound. 4-aminoquinaldinium dodecyl acetate (I) (Caldwell and others, 1961) solubility in water, 1 in 2 at 20°. In all experiments the concentration has been expressed as μg./ml. of the acetate salt.



(I)

Microbial cultures. *Staphylococcus aureus* CN 491, originally from the Wellcome collection; phage types 47, 53, 73 and 80 were obtained from the Staphylococcal Reference Laboratory, Colindale and the antibiotic resistant strains from the Luton and Dunstable Hospital; the FDA 209 strain was obtained from the National Collection of Type Cultures.

The strain of *Streptococcus viridans* was isolated at St. Bartholomew's Hospital; the strains of *Str. agalactiae* and *Dermatophilus dermatonomus* were received from the M.A.F.F. station at Weybridge. The strain of *Proteus vulgaris* (LH.14) was originally isolated at the London Hospital;

* Present address: School of Pharmacy, Faculty of Medicine, University of Khartoum.

† Registered trade mark.

the strains of *Escherichia coli* and *Bacillus subtilis* were isolated in the Bacteriological Department of Allen & Hanburys Limited. The origins and sources of all other bacterial strains used are indicated in Table I.

The fungal strains were originally from St. John's Hospital for Diseases of the Skin, except *Microsporum canis*, which was supplied by Glaxo Laboratories and *Saccharomyces cerevisiae*, which was obtained from the Distillers Company Limited. The culture of *Trichomonas vaginalis* was obtained from St. Thomas's Hospital.

TABLE I

INHIBITORY ACTION OF LAUROLINIUM ACETATE *in vitro*

Micro-organism	Strain	Geometric mean M.I.C. μg./ml. at 24 hr.*
Gram-positive bacteria		
Bacillus megatherium	KM	1·7
B. subtilis		0·2
Clostridium welchii	NCTC.8237	2·5 (48 hr.)
Corynebacterium ovis	S153	6·2
Diplococcus pneumoniae	NCTC.7465	1·5
Lactobacillus acidophilus	NCTC.2949	2·2
L. brevis	NIRD.23	6·25
Sarcina lutea	NCTC.611	0·1
Staphylococcus aureus	CN.491	0·15
Staph. aureus	phage types: 47, 53, 73, 80	0·1–0·4
Staph. aureus (antibiotic resistant)	12544,† 13588,§ 12655††	0·15–0·6
Staph. saprophyticus	NCTC.7292	0·2
Streptococcus agalactiae		0·4
Str. faecalis	R(H)	1·5
Str. pyogenes	Group C CN.771	3·1
Str. viridans	Eden	0·35
Gram-negative bacteria		
Brucella abortus	S19	3·1 (48 hr.)
Escherichia coli		8·8
E. coli	NCIB.8242	6·2
Haemophilus influenzae	NCTC.8468	12·5
Klebsiella pneumoniae	NCTC.8892	17·7
Moraxella bovis	NCTC.9425	6·2
Proteus vulgaris	LH.14	>100
Pseudomonas pyocyanea	NCTC.8203	50
Salmonella dublin	98	8·8
Salmonella typhi	NCTC.786	1·56
S. typhimurium	305	12·5
Shigella flexneri	NCTC.4832	1·56
Actinomycetes		
Actinomyces bovis	NCTC.4500	>50
Dermatophilus dermatonomus	V 2020 C	0·5
Fungi and Yeast-like fungi		
Candida albicans	1549	1·25 (3 days)
Microsporum audouini	D620	0·5 (7 days)
M. canis	764E	6·25 (7 days)
Saccharomyces cerevisiae		8·8 (3 days)
Trichophyton mentagrophytes		5·0
Protozoa		
Trichomonas vaginalis		15·0 (48 hr.)
Mycoplasma hominis (PPLO)	H32515	50

† Resistant to benzylpenicillin 5 units, chloramphenicol 50 μg., tetracycline 50 μg.
†† Resistant to benzylpenicillin 5 units, streptomycin 25 μg.
§ Resistant to benzylpenicillin 5 units, streptomycin 25 μg., tetracycline 50 μg.
* Other times of incubation are shown in brackets.

TESTS FOR BACTERIOSTATIC AND FUNGISTATIC ACTIVITY

The growth inhibitory activity of laurolinium acetate was determined *in vitro* against bacterial and fungal species using the tube dilution methods described previously (Babbs and others, 1956; Collier and others, 1959; Caldwell and others, 1961). The culture medium used for the anti-bacterial studies was dextrose-peptone water (0·5 per cent dextrose, 1·0

per cent Bacto peptone and 0·5 per cent sodium chloride in distilled water; pH 7·2), this was supplemented with 10 per cent horse serum for the culture of *Str. agalactiae*, *Str. pyogenes*, *Moraxella bovis* and *Actinomyces bovis*. *Dermatophilus dermatonomus* was cultured in nutrient broth, and in the fungistatic experiments, the dermatophytes, *Candida albicans* and *Saccharomyces cerevisiae* were grown in Sabouraud's broth.

Trichomonas vaginalis was cultured in a modified Feinberg's medium; after inoculation with a 2 day old culture of the organism, serial dilutions of laurolinium acetate in media were incubated at 37° for 48 hr. The minimal inhibitory concentration (M.I.C.) was determined microscopically.

Tests for Bactericidal Activity

General method. The bactericidal action of laurolinium acetate was investigated on washed bacterial cell suspensions at 20° using the technique described by Caldwell and others (1961).

Modified Weber and Black method. Laurolinium acetate was also examined for its bactericidal potency using the method of Chambers (1956). Its activity at 200 μg./ml. in distilled water and in synthetic hard water (200, 400 and 600 p.p.m. $CaCO_3$), was determined against heavy suspensions of *Staph. aureus* FDA 209 and *E. coli* ATCC 11229. The percentage reduction in survival of the bacteria was estimated after drug-bacteria contact times of 30 sec. and 1 min.

Results

Antimicrobial Action in vitro

The results of growth inhibitory experiments are summarised in Table I and bactericidal activity in Tables II-IV.

TABLE II

Bactericidal activity of laurolinium acetate against representative gram-positive and gram-negative bacteria

Log. Nos. of viable organisms/ml. after exposure to 90 μg./ml. for 2·5 min. at 20° C. compared with controls.

Micro-organism	Viable count		Reduction in viability per cent
	Control	Laurolinium	
Sarcina lutea	7·36	3·30	99·991
Staphylococcus aureus (CN.491)	8·00	2·65	99·999
Staph. saprophyticus	8·10	1·70	99·999
Streptococcus viridans	7·90	5·30	99·700
Escherichia coli (lab. strain)	8·23	4·70	99·960
Proteus vulgaris	8·53	4·40	99·992
Pseudomonas pyocyanea	8·25	3·10	99·999

Antagonism studies. The results of exploring possible antagonists of laurolinium acetate are expressed in Table V.

The inactivation of the compound by serum occurs as a result of the combination with serum proteins. Using 0·1 to 2·0 per cent solutions in the presence of 50 per cent serum, the laurolinium acetate-protein precipitate formed has been separated from the supernatant liquid, and both fractions found to have antibacterial activity; that of the protein

precipitate increases at the higher concentrations of the compound studied (1·0 and 2·0 per cent) where it appears that laurolinium acetate is only loosely bound to the protein.

TABLE III

BACTERICIDAL ACTIVITY OF LAUROLINIUM ACETATE AGAINST PHAGE TYPED STRAINS OF *Staphylococcus aureus** COMMONLY ASSOCIATED WITH ANTIBIOTIC RESISTANCE

Culture strain of *Staphylococcus aureus*	Concentration of laurolinium, μg./ml.	Contact time. min.	Reduction in No. of viable bacteria, per cent
CN.491	20	2·5	99·999
	80	2·5	>99·999
	100	0·5	>99·999
		1·0	>99·999
		5·0	>99·999
Phage type 47	20	2·5	99·999
59.12867	80	2·5	100
Phage type 53	20	2·5	99·999
59.13085	80	2·5	100
Phage type 73	20	2·5	99·999
59.13241	80	2·5	100
Phage type 80	20	2·5	99·998
59.13218	80	2·5	99·999

* Bacterial suspension 10^8–10^9 micro-organisms/ml.

Therapeutic Action

Antibacterial action in vivo. Laurolinium acetate was examined for its therapeutic action in mice infected intraperitoneally with a lethal inoculum of *Staph. aureus* 663. Doses of 1, 2, 4 and 8 mg./kg., injected intraperitoneally 30 min. after the bacterial culture, failed to show any protective effects; it was not possible to examine the effects of higher doses because of their toxicity.

TABLE IV

BACTERICIDAL ACTION OF LAUROLINIUM ACETATE EVALUATED BY CHAMBERS MODIFICATION OF THE WEBER AND BLACK TEST

Micro-organism	Contact time, sec.	Reduction in No. of viable bacteria per cent by laurolinium 200 μg./ml. Water hardness (p.p.m. $CaCO_3$)			
		0	200	400	500
Staphylococcus aureus FDA.209	30	99·999	99·999	99·999	95·970
	60	99·999	99·999	99·999	95·980
Escherichia coli ATCC.11229	30	99·999	99·999	99·970	98·000
	60	99·999	99·996	99·970	99·700

Sterilisation of human skin. Areas of approximately 4 cm.^{2} were marked on the inner fore-arms; in each volunteer one such area was used as a control and was swabbed with culture medium (glucose-nutrient broth with 10 per cent horse serum and 2 per cent Lubrol W added); the other skin areas were treated with aqueous and ethanolic solutions of laurolinium acetate and also ethanol alone as a further control. After drying for 2 min. the treated skin areas were swabbed with the sterile broth; swabs were incubated in the culture medium for 48 hr. at 37°. Table VI summarises the results.

Laurolinium acetate shows good activity as a skin antiseptic, both in aqueous and in 70 per cent ethanolic solution. Concentrations of 1 per cent are active against the normal bacterial flora of the skin, and of 5 per cent produce efficient sterilisation except against saprophytic spore bearing bacteria.

TABLE V

ANTAGONISM OF THE BACTERIOSTATIC ACTION OF LAUROLINIUM ACETATE AGAINST *Staphylococcus aureus* CN.491

Antagonist	Concentration, per cent	Laurolinium. Geometric mean M.I.C. in μg./ml. at 24 hr.
None		0·14
Horse serum	1	0·39
	10	6·25
	50	12·5
Saliva	10	0·19
Milk	1	1·56
	10	12·5
Pus	1	6·25
	10	100
Lecithin	0·1	50
	1	100
	2	>100
Oxgall	1	100
	2	>100
Lubrol W	1	50
	2	100
Starch (soluble)	1	0·15
	5	0·39
	10	25
Talc	10	125
Agar	0·5	0·16
	1	1·6
	2	2·2
Liquid soap	0·01	0·32
Teepol	0·01 10	<0·14
Sodium stearate	0·01	50
	0·1	>100
Sodium palmitate	0·01	>100
	0·1	>100

Similar experiments showed that human skin areas, previously contaminated with cultures of *Proteus vulgaris* and *Pseudomonas pyocyanea* were effectively sterilised by 1 and 5 per cent aqueous solutions (Table VII).

Laurodin in 5 per cent aqueous solution did not produce any skin sensitivity, allergy or photosensitisation when applied twice daily for two weeks to shaven areas of human fore-arm and rabbit skin, nor was there

TABLE VI

ANTISEPTIC ACTIVITY OF LAUROLINIUM ACETATE AGAINST NORMAL HUMAN SKIN FLORA

Solutions	No. of positive swab cultures after incubation at 37° C. for 48 hr., expressed as the ratio of the total No. of swabs taken	
	Ratio of No. of positive swabs to total No. of swabs	Ratio of No. of positive swabs to total No. of swabs excluding aerobic spore formers
Control	1·0	0·89
Laurolinium in aqueous solution—1 per cent	0·68	0·36
5 per cent	0·52	0·09
70 per cent ethanol	1·0	0·83
Laurolinium in 70 per cent ethanol—1 per cent	0·66	0·33
5 per cent	0·14	0

any pain or smarting when the solution was applied to small cuts, abrasions and burns.

Both 1 and 5 per cent solutions were also applied twice daily for one week to wounds made by a scalpel incision (1 cm. long and 0·25 cm. deep), and to lacerations (1 cm. square) made by rubbing with coarse grade sandpaper, on the shaven flanks of guinea-pigs. The 5 per cent solution slightly delayed the healing of the scalpel wounds but did not adversely affect the healing of the lacerations; the 1 per cent solution had no delaying effect.

TABLE VII

ANTISEPTIC ACTIVITY OF LAUROLINIUM ACETATE ON HUMAN SKIN AREAS PREVIOUSLY CONTAMINATED WITH PATHOGENIC BACTERIA

Solutions	No. of positive cultures of the pathogenic strains after incubation at 37° C. for 48 hr.	
	Proteus vulgaris treated areas	*Pseudomonas pyocyanea* treated areas
Control	6/6	6/6
Laurolinium in aqueous solution—5 per cent	1/6	1/6
1 per cent	0/6	0/6
0·5 per cent	1/6	3/6

Adsorption on human hair. Samples of human hair (5 mg.) in aqueous solutions of laurolinium acetate (0·1, 1·0 and 5·0 per cent) for various times, were removed, blotted and rinsed once in sterile distilled water, and placed in peptone water for 30 min. The amount of the compound released was assayed microbiologically using *Staph. aureus* CN 491.

TABLE VIII

REVERSIBLE ADSORPTION OF LAUROLINIUM ACETATE BY HUMAN HAIR*

Laurolinium concentration, per cent	Laurolinium-hair contact time, min.	Laurolinium reversibly absorbed μg./mg. hair
0·1	10	1·8
	30	1·9
	60	0·9
1·0	10	1·0
	30	0·6
	60	0·5
5·0	10	0·9
	30	0·4
	60	0·3

* Laurolinium assayed microbiologically using *Staph. aureus* CN.491 as the test organism.

Appreciable concentrations of the compound are reversibly adsorbed on to human hair (Table VIII); the maximum adsorption taking place from the most dilute solution (0·1 per cent) after 10 min. contact. Higher concentrations and contact times beyond 10 min. did not result in increased adsorption.

Toxicology and General Pharmacology

Quaternary ammonium compounds are noted for their diversity of biological action; a study was therefore made of the toxicology and general

pharmacology of laurolinium acetate to see if some indication of the cause of its high parenteral toxicity could be found.

Toxicity. In acute studies in mice, the LD50 values (mg./kg.) were: oral 131·8 ± 36·2; subcutaneous 30·2 ± 5·6; intraperitoneal 2·3 ± 0·2 and intravenous 6·0 ± 0·4; mice showed marked symptoms of central nervous system depression after the intravenous injection of toxic doses. Given orally to rats in sub-acute tests, it was not toxic at a dose of 8 mg./kg. daily for 6 weeks; doses of 20 and 50 mg./kg. over the same period were toxic although blood examination showed no difference between treated and control animals.

Solutions of laurolinium acetate, in sterile distilled water (saline caused precipitation), were examined for their irritant action in the rabbit eye; 0·01 per cent was non-irritant when instilled into the eye, twice daily for 1 week; 0·1 per cent produced slight irritation and 1·0 and 5·0 per cent produced severe irritation with obvious distress to the animals.

Pharmacology. Laurolinium acetate, in doses extending to toxic levels, was devoid of any neuromuscular blocking activity, either by a curare-like or a depolarising action, nor was there any evidence of ganglionic blocking, anticholinesterase or antihistaminic activity. When injected intravenously, it had no direct effect on cardiac rhythm; however, it produced transient hypotension followed by prolonged hypertension. Large intravenous doses produced salivation, lachrymation and increased tracheal mucous secretion in the cat; these effects were not antagonised by pretreatment with atropine. In *in vitro* studies on isolated tissues, high doses appeared to be cytotoxic.

Discussion

Laurolinium acetate shows a wide *in vitro* antimicrobial activity; it is effective against representative Gram-positive and Gram-negative bacteria, against many pathogenic fungi and *Trichomonas vaginalis* and *Mycoplasma hominis* ('PPLOS').

Caldwell and others (1961) have demonstrated it to be one of the most active members of its chemical series. A comparison between the activity of this quaternary and the allied decamethylene-bis-4-aminoquinaldinium acetate (dequalinium) (Babbs and others, 1956), which has proved highly successful as a topical anti-infective agent (Wilkinson, 1959), has shown a similar range of bacteriostatic activity against Gram-positive bacteria and a closely related, although slightly lower activity against Gram-negative bacteria; the antifungal activity of the two agents is also very similar. The significant difference between the mono and bis quaternary ammonium compound is that unlike dequalinium, laurolinium acetate has, in addition to its other properties, a rapid and potent bactericidal activity which is more pronounced against Gram-positive bacteria.

With *Staphylococcus aureus*, the bacteriostatic and bactericidal activity of laurolinium acetate is independent of strain differences; thus it is equally effective against the laboratory strain of *Staph. aureus* CN 491,

the phage types 47, 53, 73 and 80, and against strains resistant to benzylpenicillin and other antibiotics. This lack of strain specificity in its action is good supportive evidence that, like other cationic antibacterial agents, it combines with specific anionic sites in the bacterial cell.

The efficacy of laurolinium acetate as a bactericide in Chambers modification of the Weber and Black test (Chambers, 1956) is well shown by the almost complete sterilisation (99·999 per cent) of cultures of *Staph. aureus* and *E. coli*, by a concentration of 200 μg./ml. in contact for 30 sec. Bactericidal activity decreases in the presence of increasing water hardness although this is more evident against *E. coli* than against *Staph. aureus*. This method of evaluating cationic bactericides, although not common in this country, is included in the recommended methods of the United States Public Health Service and the U.S. Department of Agriculture.

As with most quaternary ammonium compounds, its antibacterial action is antagonised by organic matter and by anionic substances. The antagonism shown by the soaps (but not potash soft soap) and bile salts is common to many cationic bactericides. It is not antagonised to any great extent by saliva, but there is some antagonism by milk, pus and serum. Serum antagonism is due to precipitation of the serum proteins; however, the compound is only loosely bound to the precipitate. In the presence of a slight excess of laurolinium acetate the compound-protein precipitate retains antibacterial activity. In similar studies Van Eseltine and Hucker (1948) showed that a closely related quaternary salt—laurylisoquinolinium bromide was less antagonised in its antibacterial action in the presence of serum, milk, cotton-seed-oil and soluble starch, than were a number of other aliphatic quaternary ammonium compounds in use at that time.

The rapid bactericidal activity of laurolinium acetate in *in vitro* experiments suggested that solutions of the compound might be used to sterilise the skin and its appendages. In subsequent tests on human volunteers swabbing the fore-arm with 5·0 and 1·0 per cent solutions in water or 70 per cent ethanol reduced the normal skin flora. Related studies to examine the activity of solutions on intact skin areas, previously contaminated with cultures of *Proteus vulgaris* and *Pseudomonas pyocyanea*, also showed its sterilising ability. The results from adsorption studies using human hair showed that the compound was well adsorbed over a wide range of concentrations; and local toxicity studies showed it to be free from untoward effects on intact and abrased skin, even though repeated applications of a 5 per cent solution were made. In the clinic, Verdon (1961) has made a comparative survey of the efficacy of a series of common skin antiseptics under ward conditions; the results of which so favoured the use of a 5 per cent solution of laurolinium acetate that this has since been adopted as the standard pre-injection skin disinfectant in all wards of the Portsmouth Group of Hospitals.

From the present study it is apparent that laurolinium acetate is suitable for use in the treatment of local infective conditions, for the sterilisation of skin areas and for general antiseptic purposes. Unfortunately its toxicity precludes its systemic use and its basic pharmacology

suggests that the systemic toxicity is related to protein precipitating activity.

Acknowledgements. We would like to thank Miss J. A. Moore and Miss C. A. Bedford for their assistance with the antimicrobial experiments, Mrs. J. E. Baldwin for investigating the Pharmacology of laurolinium acetate and Mr. N. W. Spurling for the toxicological studies.

References

Babbs, M., Collier, H. O. J., Austin, W. C., Potter, M. D. and Taylor, E. P. (1956). *J. Pharm. Pharmacol.*, **8**, 110–119.
Caldwell, D., Cox, W. A., D'Arcy, P. F. and Rowe, L. R. (1961). *Ibid.*, **13**, 554–564.
Chambers, C. W. (1956). *J. Milk and Food Technol.*, **19**, 183–187.
Collier, H. O. J., Cox, W. A., Huskinson, P. L. and Robinson, F. A. (1959). *J. Pharm. Pharmacol.*, **11**, 671–680.
Cox, W. A. and D'Arcy, P. F. (1959). *J. gen. Microbiol.*, **27**, i-ii.
Cox, W. A. and D'Arcy, P. F. (1961). *J. Pharm. Pharmacol.*, **13**, 34–38.
Van Eseltine, W. P. and Hucker, G. J. (1948). *N.Y. State Agr. Exp. Sta. Tech. Bull.*, **282**, 3–20.
Verdon, P. E. (1961). *J. clin. Path.*, **14**, 91–93.
Wilkinson, D. S. (1959). *Practitioner*, **182**, 501–506.

STUDIES IN THE FIELD OF DIURETIC AGENTS

PART VII. 4-CHLORO-2′-METHYL-3-SULPHAMOYLBENZANILIDE

BY V. PETROW, O. STEPHENSON AND A. M. WILD

*From the British Drug Houses, Ltd., Graham Street, London, N.*1

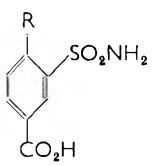
Received August 14, 1962

A new route to the anilides of 4-halogeno-3-sulphamoylbenzoic acid is described. These novel compounds, and in particular 4-chloro-2′-methyl-3-sulphamoylbenzanilide, are found to be potent orally active diuretic agents.

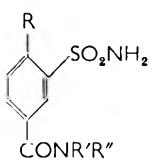
IN Part VI of this series (Jackman, Petrow, Stephenson and Wild, 1962) the preparation was described of some 4-substituted 3-sulphamoylbenzoic acids, a new class of orally active diuretic agents with only slight inhibitory activity against carbonic anhydrase. Such compounds (I) showed optimal activity when the substituent R was a fluoro-, chloro- or bromo-atom. The present study deals with some related carboxyamides (II).

Work was originally confined to the preparation of the more readily available 4-chloro-compounds (II: R = Cl), but, following early promising biological results, was later extended to analogous types.

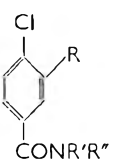
The unsubstituted amide (II; R = Cl; R′ = R″ = H) was obtained readily from 4-chloro-3-chlorosulphonylbenzoyl chloride, itself prepared by a variation of the method described by Ullmann in 1896. For the preparation of substituted carboxyamides, recourse was made to 4-chloro-3-nitrobenzoyl chloride, described by Montagne in 1900. This was condensed with the appropriate amine R′R″NH to yield the 4-chloro-3-nitrobenzamide (III; R = NO_2), which was in turn reduced to the corresponding aniline (III; R = NH_2). The last compound was then converted into the required sulphonamide (II; R = Cl) *via* the sulphonchloride (III; R = SO_2Cl) as described by Petrow, Stephenson and Wild (1960). (Data for amino and nitro-compounds are in Table I.)



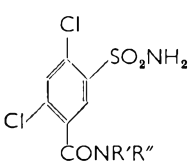
(I)



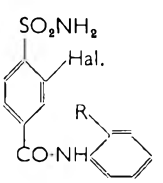
(II)



(III)



(IV)



(V)

The methyl-, ethyl-, and dimethyl-carboxyamides were all prepared by this route. They resembled the unsubstituted amide in being effective

oral diuretic agents at the 5 mg./kg. dose level in saline-loaded rats in tests carried out by Dr. A. David and his colleagues.

Extension of this work to the benzanilide derivatives (II; R = Cl, R′ = H, R″ = aryl) led to a major advance in this field. 4-Chloro-2′-methyl-3-sulphamoylbenzanilide (II; R = Cl, R′ = H, R″ = *o*-tolyl), prepared by the method described, proved to be an extremely potent oral diuretic agent with an activity fifty to one hundred times that of the parent acid (I; R = Cl). This important result led to a study of improved methods for the preparation of these compounds. The most satisfactory route discovered depends upon the greater reactivity of the carboxylyl chloride group relative to the sulphonchloride group [compare Wegscheider and Furcht (1902); Smiles and Stewart (1921) and Barr, Salminen and Weissberger (1951)]. Thus we found that 4-chloro-3-chlorosulphonylbenzoyl chloride condensed readily with one equivalent of *o*-toluidine, or its hydrochloride, in boiling toluene or chlorobenzene to give a 90 per cent yield of 4-chloro-3-chlorosulphonyl-2′-methylbenzanilide (III; R = SO_2Cl, R′ = H, R″ = *o*-tolyl). The reaction was applied to a whole range of amines including substituted anilines. The sulphonchlorides prepared in this way are summarised in Table II. Reaction of these sulphonchlorides with ammonia or amines furnished the required sulphonamides (Table III) in high yield.

Biological testing of the sulphonamides by Dr. A. David and his colleagues showed that the high oral diuretic activity of the *o*-toluidide was possessed by several other *ortho*-substituted analogues, the 4-chloro-2′-halogeno-3-sulphamoylbenzanilides (II; R = Cl, R′ = H, R″ = *o*-F-, -Cl- or -Br-C_6H_4) being especially active. The corresponding *meta*-(3′-) and *para*- (4′-) substituted compounds were much less active, as was the compound unsubstituted in the aniline ring (II; R = Cl, R′ = H, R″ = Ph). In the last compound, activity was increased by the introduction of a methyl substituent into the carboxyamide, as in 4-chloro-*N*-methyl-3-sulphamoylbenzanilide (II; R = Cl, R′ = Me, R″ = Ph), but in general an additional alkyl substituent in the carboxyamide caused no increase in diuretic activity.

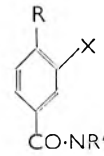
2′-Carboxy-4-chloro-3-sulphamoylbenzanilide (II; R = Cl, R′ = H, R″ = *o*-$CO_2H{\cdot}C_6H_4$), a possible metabolite of the active *o*-toluidide, was prepared by reaction of 4-chloro-3-chlorosulphonylbenzoyl chloride with anthranilic acid or with methyl anthranilate, followed in the latter case by hydrolysis of the ester. It proved to be without diuretic activity.

The introduction of further substituents into the aniline ring of the benzanilide nucleus caused no apparent increase in diuretic activity, though the more active of these types all possessed an *ortho*-substituent.

Fluoro- and bromo-analogues (II; R = F or Br, R′ = H, R″ = aryl) of the more potent compounds of the chloro-series were prepared by similar reaction techniques and proved to be highly active oral diuretic agents. The corresponding compounds (II; R = Me, R′ = H, R″ = aryl) derived from 4-methyl-3-sulphamoylbenzoic acid were somewhat less effective.

TABLE I

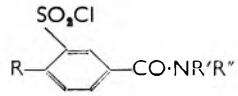
AMINO- AND NITRO-COMPOUNDS



R	X	R′	R″	m.p. °C	Formula	Found				Required			
						C	H	Cl	N	C	H	Cl	N
Cl	NH_2	H	Me	148–149	$C_8H_9ClN_2O$	52·3	4·7	18·9	15·4	52·1	4·9	19·2	15·2
Cl	NO_2	H	Et	101	$C_9H_9ClN_2O_3$	47·2	4·1	15·3	12·4	47·2	4·0	15·5	12·3
Cl	NH_2	H	Et	106–108	$C_9H_{11}ClN_2O$	54·5	5·7	17·8	14·0	54·4	5·6	17·8	14·1
Cl	NO_2	H	*o*-Me·C_6H_4	151–153	$C_{14}H_{11}ClN_2O_3$	57·9	3·8	11·9	10·0	57·8	3·8	12·2	9·6
Cl	NH_2	H	*o*-Me·C_6H_4	160–162	$C_{14}H_{13}ClN_2O$	64·4	5·1	13·7	10·9	64·5	5·0	13·6	10·7
Cl	NO_2	H	Ph·CH_2	99–101	$C_{14}H_{11}ClN_2O_3$	57·8	4·3	12·2	9·5	57·8	3·8	12·2	9·6
Cl	NH_2·HCl	H	Ph·CH_2	192–194	$C_{14}H_{14}Cl_2N_2O$	54·1	5·0	24·0	9·7	54·3	4·8	23·8	9·4
Cl	NO_2	H	Ph·CH_2·CH_2	95–97	$C_{15}H_{13}ClN_2O_3$	59·1	4·7	11·4	8·9	59·1	4·3	11·6	9·2
Cl	NH_2	H	Ph·CH_2·CH_2	134–135	$C_{15}H_{15}ClN_2O$	65·7	5·3	—	9·9	65·6	5·5	12·9	10·2
Cl	NH_2	Me	Me	73–75	$C_9H_{11}ClN_2O$	54·8	5·3	18·2	14·0	54·4	5·6	17·8	14·1
Cl	NO_2	$-[CH_2]_4-$		79–81	$C_{10}H_{11}ClN_2O_3$	—	—	13·1	10·1	—	—	13·2	10·4
Cl	NH_2	$-[CH_2]_5-$		141–143	$C_{12}H_{15}ClN_2O$	60·8	6·5	14·7	11·6	60·4	6·3	14·9	11·7
Me	NH_2·HCl	Me	Me	221–223	$C_{10}H_{15}ClN_2O$	55·7	7·3	16·4	12·8	55·9	7·0	16·5	13·1

TABLE II

SULPHONCHLORIDES

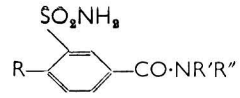


R	R′	R″	m.p. °C	Formula	Found C	Found H	Found Cl	Found N	Found S	Required C	Required H	Required Cl	Required N	Required S
Me	H	*o*-Me·C_6H_4	128–130	$C_{15}H_{14}ClNO_3S$	55·8	4·5	10·6	4·2	9·8	55·5	4·4	10·9	4·3	9·9
Me	H	*o*-Cl·C_6H_4	137–138	$C_{14}H_{11}Cl_2NO_3S$	48·8	3·1	20·6	4·1	9·5	48·7	3·2	20·5	4·1	9·3
Me	H	2,5-Cl_2·C_6H_3	136–137	$C_{14}H_{10}Cl_3NO_3S$	44·6	3·0	27·8	3·7	8·6	44·4	2·7	28·1	3·7	8·5
F	H	*o*-Me·C_6H_4	163	$C_{14}H_{11}ClFNO_3S$	51·2	3·3	10·9	4·0	10·2	51·1	3·4	10·8	4·3	9·8
F	H	*o*-F·C_6H_4	140–141	$C_{13}H_8ClF_2NO_3S$	47·1	2·6	—	4·4	9·6	47·1	2·4	—	4·2	9·7
F	H	*o*-Cl·C_6H_4	112–113	$C_{13}H_8Cl_2FNO_3S$	45·1	2·6	19·9	4·0	—	44·8	2·3	20·4	4·0	—
F	H	*o*-Br·C_6H_4	124–125	$C_{13}H_8BrClFNO_3S$	40·2	2·2	—	3·8	—	39·8	2·1	—	3·6	—
F	H	2,5-Cl_2·C_6H_3	145–146	$C_{13}H_7Cl_2FNO_3S$	40·9	2·2	—	4·1	—	40·8	1·8	—	3·7	—
Cl	H	Me	114–116	$C_8H_7Cl_2NO_3S$	—	—	—	5·1	11·7	—	—	—	5·2	12·0
Cl	H	Pr^i	117–119	$C_{10}H_{11}Cl_2NO_3S$	—	—	—	4·7	10·6	—	—	—	4·7	10·8
Cl	H	Ph	198	$C_{13}H_9Cl_2NO_3S$	47·2	2·5	—	4·0	9·7	47·3	2·8	—	4·2	9·7
Cl	H	*o*-Me·C_6H_4	161–163	$C_{14}H_{11}Cl_2NO_3S$	49·0	3·3	20·3	4·2	9·0	48·8	3·2	20·6	4·1	9·3
Cl	H	*m*-Me·C_6H_4	174–176	$C_{14}H_{11}Cl_2NO_3S$	49·3	3·0	20·7	4·3	9·6	48·8	3·2	20·6	4·1	9·3
Cl	H	*p*-Me·C_6H_4	168–169	$C_{14}H_{11}Cl_2NO_3S$	49·1	3·1	21·2	4·2	9·5	48·8	3·2	20·6	4·1	9·3
Cl	H	*o*-MeO·C_6H_4	144–146	$C_{14}H_{11}Cl_2NO_4S$	46·9	2·6	20·2	3·8	—	46·7	2·9	19·7	3·9	—
Cl	H	*o*-F·C_6H_4	156–157	$C_{13}H_8Cl_2FNO_3S$	45·2	2·5	—	4·0	9·2	44·8	2·3	—	4·0	9·2
Cl	H	*o*-Cl·C_6H_4	146–148	$C_{13}H_8Cl_3NO_3S$	—	—	29·1	3·8	9·1	—	—	29·2	3·8	8·8
Cl	H	*m*-Cl·C_6H_4	194	$C_{13}H_8Cl_3NO_3S$	43·2	2·1	28·6	3·6	8·6	42·8	2·2	29·2	3·8	8·8
Cl	H	*p*-Cl·C_6H_4	177–178	$C_{13}H_8Cl_3NO_3S$	43·2	2·1	29·6	3·7	8·8	42·8	2·2	29·2	3·8	8·8
Cl	H	*o*-Br·C_6H_4	145–146	$C_{13}H_8BrCl_2NO_3S$	38·5	2·1	37·2*	3·6	8·2	38·2	2·0	36·9*	3·4	7·8
Cl	H	*m*-CF_3·C_6H_4	144–146	$C_{14}H_8Cl_2F_3NO_3S$	41·9	2·1	—	3·8	—	42·2	2·0	—	3·5	—
Cl	H	*o*-MeO·CO·C_6H_4	149–151	$C_{15}H_{11}Cl_2NO_5S$	46·7	2·9	18·7	4·1	8·5	46·4	2·9	18·3	3·6	8·3
Cl	H	2,3-Me_2·C_6H_3	173–174	$C_{15}H_{13}Cl_2NO_3S$	50·2	3·7	—	3·9	8·6	50·3	3·7	—	3·9	9·0
Cl	H	2,4-Me_2·C_6H_3	158–160	$C_{15}H_{13}Cl_2NO_3S$	50·1	3·3	19·8	3·9	9·4	50·3	3·7	19·8	3·9	9·0
Cl	H	2,5-Me_2·C_6H_3	190–191	$C_{15}H_{13}Cl_2NO_3S$	50·5	3·5	20·0	4·3	9·3	50·3	3·7	19·8	3·9	9·0
Cl	H	2,6-Me_2·C_6H_3	185–186	$C_{15}H_{13}Cl_2NO_3S$	50·6	3·6	20·4	—	9·1	50·3	3·7	19·8	—	9·0
Cl	H	2,3-Cl_2·C_6H_3	132–134	$C_{13}H_7Cl_4NO_3S$	39·5	1·8	35·5	3·6	8·0	39·1	1·8	35·5	3·5	8·0
Cl	H	2,4-Cl_2·C_6H_3	151–153	$C_{13}H_7Cl_4NO_3S$	—	—	35·4	3·5	8·4	—	—	35·5	3·5	8·0
Cl	H	2,5-Cl_2·C_6H_3	155–156	$C_{13}H_7Cl_4NO_3S$	39·5	1·6	35·6	—	8·2	39·1	1·8	35·5	—	8·0
Cl	H	2,6-Cl_2·C_6H_3	210	$C_{13}H_7Cl_4NO_3S$	—	—	35·6	3·5	8·3	—	—	35·5	3·5	8·0
Cl	H	2,6-Br_2·C_6H_3	223–225	$C_{13}H_7Br_2Cl_2NO_3S$	32·1	1·4	47·3*	3·0	6·8	32·0	1·5	47·3*	2·9	6·6
Cl	H	2,4,5-Cl_3·C_6H_2	157–158	$C_{13}H_6Cl_5NO_3S$	36·4	1·4	40·7	3·6	7·4	36·0	1·4	40·9	3·2	7·4
Cl	H	2,4,6-Cl_3·C_6H_2	177–179	$C_{13}H_6Cl_5NO_3S$	36·5	1·2	40·3	3·2	7·4	36·0	1·4	40·9	3·2	7·4
Cl	H	Ph·CH_2	152–154	$C_{14}H_{11}Cl_2NO_3S$	49·3	3·0	20·2	4·4	9·3	48·8	3·2	20·6	4·1	9·3
Cl	Me	Me	106–108	$C_9H_9Cl_2NO_3S$	38·3	3·0	24·9	5·2	11·3	38·3	3·2	25·1	5·0	11·4
Cl	Me	Ph	120–122	$C_{14}H_{11}Cl_2NO_3S$	49·1	3·2	21·2	4·2	9·8	48·8	3·2	20·6	4·1	9·3
Cl	Me	*o*-Me·C_6H_4	119–121	$C_{15}H_{13}Cl_2NO_3S$	50·5	3·5	—	4·4	—	50·3	3·7	—	3·9	—
Cl	Et	Ph	126–128	$C_{15}H_{13}Cl_2NO_3S$	50·4	3·9	20·0	3·9	9·2	50·3	3·7	19·8	3·9	9·0
Cl	[CH_2]$_5$–		117–119	$C_{12}H_{15}Cl_2NO_3S$	44·7	4·1	22·4	4·1	10·1	44·7	4·1	22·0	4·4	10·0
Br	H	*o*-Me·C_6H_4	170–171	$C_{14}H_{11}BrClNO_3S$	43·0	2·9	—	3·8	8·4	43·1	2·8	—	3·6	8·2
Br	H	*o*-Cl·C_6H_4	166–167	$C_{13}H_8BrCl_2NO_3S$	38·2	1·8	—	3·6	7·6	38·2	2·0	—	3·4	7·8
Br	H	2,5-Cl_2·C_6H_3	163–164	$C_{13}H_7BrCl_3NO_3S$	35·4	1·5	—	3·1	7·0	35·2	1·6	—	3·2	7·2

* Total Halogen.

TABLE III

SULPHONAMIDES



R	R′	R″	m.p. °C	Formula	Found C	Found H	Found Cl	Found N	Found S	Required C	Required H	Required Cl	Required N	Required S
H	H	H	170	$C_7H_8N_2O_3S$	—	—	—	—	—	—	—	—	—	—
H	H	Me	212–214	$C_8H_{10}N_2O_3S$	45·1	4·6	—	13·1	14·7	44·9	4·7	—	13·1	15·0
H	H	*o*-Me·C_6H_4	190–191	$C_{14}H_{14}N_2O_3S$	57·9	5·1	—	9·8	10·7	57·9	4·9	—	9·7	11·0
Me	H	H	219–221	$C_8H_{10}N_2O_3S$	45·1	4·8	—	13·1	14·9	44·9	4·7	—	13·1	15·0
Me	H	Me	219–221	$C_9H_{12}N_2O_3S$	47·7	5·5	—	12·0	14·0	47·4	5·3	—	12·3	14·1
Me	H	*o*-Me·C_6H_4	230–231	$C_{15}H_{16}N_2O_3S$	59·4	5·2	—	8·9	10·3	59·0	5·3	—	9·2	10·5
Me	H	*o*-Cl·C_6H_4	192–194	$C_{14}H_{13}ClN_2O_3S$	51·5	3·9	11·0	8·9	9·7	51·6	4·0	10·9	8·6	9·8
Me	H	2,5-Cl_2·C_6H_3	189–190	$C_{14}H_{12}Cl_2N_2O_3S$	47·1	3·4	19·7	7·7	8·9	46·8	3·3	19·7	7·8	8·9
Me	Me	Me	155–157	$C_{10}H_{14}N_2O_3S$	50·0	5·7	—	11·6	13·1	49·6	5·8	—	11·6	13·2
F	H	H	212–214	$C_7H_7FN_2O_3S$	38·3	3·4	—	12·9	—	38·5	3·2	—	12·8	—
F	H	*o*-Me·C_6H_4	205–206	$C_{14}H_{13}FN_2O_3S$	54·8	4·5	—	9·2	10·7	54·4	4·2	—	9·1	10·4
F	H	*o*-F·C_6H_4	213–215	$C_{13}H_{10}F_2N_2O_3S$	49·6	3·3	—	8·5	—	50·0	3·2	—	9·0	—
F	H	*o*-Cl·C_6H_4	221–223	$C_{13}H_{10}ClFN_2O_3S$	47·7	3·3	11·3	8·6	10·0	47·5	3·1	10·8	8·5	9·8
F	H	*o*-Br·C_6H_4	219–220	$C_{13}H_{10}BrFN_2O_3S$	41·7	2·8	21·3[1]	7·6	8·5	41·8	2·7	21·4[1]	7·5	8·9
F	H	2,5-Cl_2·C_6H_3	197–198	$C_{13}H_9Cl_2FN_2O_3S$	43·4	2·6	19·7	8·0	9·1	43·0	2·5	19·5	7·7	8·8
Cl	H	H	228–230	$C_7H_7ClN_2O_3S$	—	—	—	—	—	—	—	—	—	—
Cl	H	Me	230–232	$C_8H_9ClN_2O_3S$	38·8	3·6	14·4	11·4	13·3	38·6	3·7	14·3	11·3	12·9
Cl	H	Et	208–210	$C_9H_{11}ClN_2O_3S$	40·4	4·0	13·5	11·0	12·2	41·1	4·2	13·5	10·7	12·2
Cl	H	Pr^i	222–224	$C_{10}H_{13}ClN_2O_3S$	43·5	4·4	—	10·4	—	43·4	4·7	—	10·1	—
Cl	H	Ph	247–249	$C_{13}H_{11}ClN_2O_3S$	50·2	3·5	11·3	9·3	10·5	50·2	3·6	11·4	9·0	10·3
Cl	H	*o*-Me·C_6H_4	240–242	$C_{14}H_{13}ClN_2O_3S$	51·9	4·1	10·9	8·6	9·8	51·8	4·0	10·9	8·6	9·9
Cl	H	*m*-Me·C_6H_4	204–205	$C_{14}H_{13}ClN_2O_3S$	51·8	3·8	10·7	8·5	9·7	51·8	4·0	10·9	8·6	9·9
Cl	H	*p*-Me·C_6H_4	234–236	$C_{14}H_{13}ClN_2O_3S$	52·2	3·9	11·3	8·8	10·2	51·8	4·0	10·9	8·6	9·9
Cl	H	*o*-MeO·C_6H_4	214–215	$C_{14}H_{13}ClN_2O_4S$	49·5	4·0	10·7	8·1	9·2	49·3	3·8	10·4	8·2	9·4
Cl	H	*o*-F·C_6H_4	192–194	$C_{13}H_{10}ClFN_2O_3S$	47·6	3·3	11·2	9·0	9·4	47·5	3·1	10·8	8·5	9·8
Cl	H	*o*-Cl·C_6H_4	217–218	$C_{13}H_{10}Cl_2N_2O_3S$	45·5	3·1	20·8	8·3	9·5	45·2	2·9	20·5	8·1	9·3
Cl	H	*m*-Cl·C_6H_4	221–222	$C_{13}H_{10}Cl_2N_2O_3S$	45·6	2·8	—	8·2	9·4	45·2	2·9	—	8·1	9·3
Cl	H	*p*-Cl·C_6H_4	235–236	$C_{13}H_{10}Cl_2N_2O_3S$	45·6	3·1	20·8	8·0	9·5	45·2	2·9	20·5	8·1	9·3
Cl	H	*o*-Br·C_6H_4	209–211	$C_{13}H_{10}BrClN_2O_3S$	39·8	2·3	30·2[2]	7·1	8·5	40·1	2·6	29·6[2]	7·2	8·2
Cl	H	*m*-CF_3·C_6H_4	195–197	$C_{14}H_{10}ClF_3N_2O_3S$	44·4	3·0	—	7·0	—	44·4	2·7	—	7·4	—
Cl	H	*o*-CO_2H·C_6H_4	264–266 (*d*)	$C_{14}H_{11}ClN_2O_5S$	47·5	3·0	10·1	7·8	8·7	47·3	3·1	10·0	7·9	9·0
Cl	H	*o*-MeO·CO·C_6H_4	226–227	$C_{15}H_{13}ClN_2O_5S$	48·8	3·5	9·6	7·7	8·9	48·9	3·6	9·6	7·6	8·7
Cl	H	*p*-NH_2SO_2·C_6H_4	294–296	$C_{13}H_{12}ClN_3O_5S_2$	40·3	3·0	9·3	10·9	16·3	40·1	3·1	9·1	10·8	16·5
Cl	H	2,3-Me_2·C_6H_3	261–263	$C_{15}H_{16}ClN_2O_3S$	53·3	4·3	10·7	8·3	9·9	53·2	4·5	10·5	8·3	9·5
Cl	H	2,4-Me_2·C_6H_3	225–227	$C_{15}H_{15}ClN_2O_3S$	53·1	4·1	10·6	8·4	9·6	53·2	4·5	10·5	8·3	9·5
Cl	H	2,5-Me_2·C_6H_3	225–226	$C_{15}H_{16}ClN_2O_3S$	52·7	4·4	10·4	8·4	9·8	53·2	4·5	10·5	8·3	9·5
Cl	H	2,6-Me_2·C_6H_3	245–247	$C_{15}H_{16}ClN_2O_3S$	53·4	4·4	10·6	8·2	9·4	53·2	4·5	10·5	8·3	9·5

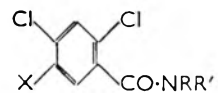
TABLE III *continued*

R	R′	R″	m.p. °C	Formula	Found C	Found H	Found Cl	Found N	Found S	Required C	Required H	Required Cl	Required N	Required S
Cl	H	2,3-Cl_2·C_6H_3	218–220	$C_{13}H_9Cl_3N_2O_3S$	41·2	2·4	27·8	7·2	8·5	41·1	2·4	28·0	7·4	8·4
Cl	H	2,4-Cl_2·C_6H_3	202–204	$C_{13}H_9Cl_3N_2O_3S$	41·3	2·1	28·1	7·4	8·5	41·1	2·4	28·0	7·4	8·4
Cl	H	2,5-Cl_2·C_6H_3	218	$C_{13}H_9Cl_3N_2O_3S$	41·0	2·5	—	7·3	8·4	41·1	2·4	—	7·4	8·4
Cl	H	2,6-Cl_2·C_6H_3	219–220	$C_{13}H_9Cl_3N_2O_3S$	41·1	2·1	28·0	7·3	8·5	41·1	2·4	28·0	7·4	8·4
Cl	H	2,6-Br_2·C_6H_3	246–248	$C_{13}H_9Br_2ClN_2O_3S$	33·1	1·6	42·3[2]	6·0	7·3	33·3	1·9	41·7[2]	6·0	6·8
Cl	H	2,4,5-Cl_3·C_6H_2	262–264	$C_{13}H_8Cl_4N_2O_3S$	37·4	1·8	34·8	6·5	7·5	37·7	2·0	34·3	6·8	7·7
Cl	H	2,4,6-Cl_3·C_6H_2	205–206	$C_{13}H_8Cl_4N_2O_3S$	38·1	1·9	34·8	6·7	7·9	37·7	2·0	34·3	6·8	7·7
Cl	H	Ph·CH_2	186–187	$C_{14}H_{12}ClN_2O_3S$	52·1	3·6	11·3	9·0	10·2	51·8	4·0	10·9	8·6	9·9
Cl	H	Ph·CH_2·CH_2	172–174	$C_{15}H_{14}ClN_2O_3S$	53·3	4·5	10·1	7·8	9·4	53·2	4·5	10·5	8·3	9·5
Cl	Me	Me	135–136	$C_9H_{11}ClN_2O_3S$	41·2	4·3	13·3	10·8	12·0	41·2	4·2	13·5	10·7	12·2
Cl	Me	Ph	194–196	$C_{14}H_{13}ClN_2O_3S$	52·2	3·9	—	—	10·3	51·8	4·0	—	—	9·9
Cl	Me	*o*-Me·C_6H_4	188–190	$C_{15}H_{15}ClN_2O_3S$	53·2	4·2	10·6	8·2	9·3	53·2	4·5	10·5	8·3	9·5
Cl	Et	Ph	167–168	$C_{15}H_{15}ClN_2O_3S$	53·8	4·3	10·6	7·9	9·3	53·2	4·5	10·5	8·3	9·5
Cl	Et	*o*-Me·C_6H_4	178–180	$C_{16}H_{17}ClN_2O_3S$	54·7	5·0	10·2	8·0	8·9	54·5	4·9	10·1	7·9	9·1
Cl	Et	*o*-Cl·C_6H_4	173–175	$C_{15}H_{14}Cl_2N_2O_3S$	48·4	3·9	18·9	7·6	8·9	48·3	3·8	19·0	7·5	8·6
Cl	$-[CH_2]_5-$		178–180	$C_{12}H_{15}ClN_2O_3S$	47·4	4·7	11·9	9·4	10·6	47·6	5·0	11·7	9·3	10·6
Br	H	*o*-Me·C_6H_4	245–246	$C_{14}H_{13}BrN_2O_3S$	45·1	3·5	—	8·0	9·1	45·4	3·5	—	7·6	8·7
Br	H	*o*-Cl·C_6H_4	228–230	$C_{13}H_{10}BrClN_2O_3S$	40·0	2·4	—	7·4	8·5	40·1	2·6	—	7·2	8·2
Br	H	2,5-Cl_2·C_6H_3	226–228	$C_{13}H_9BrCl_2N_2O_3S$	37·1	2·2	35·0[2]	6·4	7·4	36·8	2·1	35·6[2]	6·6	7·6

1 = Bromine
2 = Total Halogen

TABLE IV

DERIVATIVES OF 2,4-DICHLOROBENZOIC ACID



R	R′	X	m.p. °C	Formula	Found C	Found H	Found Cl	Found N	Found S	Required C	Required H	Required Cl	Required N	Required S
H	H	NH_2·SO_2	216–217	$C_7H_6Cl_2N_2O_3S$	—	—	26·0	10·2	12·1	—	—	26·3	10·4	11·9
H	Me	NO_2	144–145	$C_8H_6Cl_2N_2O_3$	38·7	2·5	—	—	—	38·6	2·4	—	—	—
H	Me	NH_2	137–138	$C_8H_6Cl_2N_2O$	43·6	3·8	—	12·8	—	43·9	3·7	—	12·8	—
H	Me	Cl·SO_2	174–175	$C_8H_5Cl_3NO_3S$	32·2	2·2	34·9	4·6	10·3	31·8	2·0	35·1	4·6	10·6
H	Me	NH_2·SO_2	223–225	$C_8H_7Cl_2N_2O_3S$	34·2	2·8	25·0	10·1	11·8	33·9	2·9	25·0	9·9	11·3
Me	Me	NO_2	155–157	$C_9H_8Cl_2N_2O_3$	41·4	3·1	—	—	—	41·0	3·1	—	—	—
Me	Me	NH_2	149–150	$C_9H_{10}Cl_2N_2O$	46·6	4·4	30·2	12·5	—	46·3	4·3	30·4	12·0	—
Me	Me	Cl·SO_2	156–158	$C_9H_8Cl_3NO_3S$	—	—	33·8	4·2	10·1	—	—	33·6	4·4	10·1
Me	Me	NH_2·SO_2	243–245	$C_9H_{10}Cl_2N_2O_3S$	36·8	3·4	23·5	—	11·1	36·4	3·4	23·9	—	10·8

A few amides (cf. IV) of 2,4-dichloro-5-sulphamoylbenzoic acid were also prepared (cf. Table IV), but these had no diuretic activity at a dose level of 10 mg./kg. in saline-loaded rats.

Finally, three analogues of the most active benzanilides but with the halogen and sulphamoyl groups interchanged (cf. V; R = Me or Cl) were prepared by heating the 3-halogeno-4-sulphamoylbenzoic acid with *o*-toluidine or *o*-chloroaniline at reflux temperature in an atmosphere of nitrogen. They had no diuretic activity when administered to saline-loaded rats at doses of 10 mg./kg.

Most of the compounds described were less potent inhibitors of carbonic anhydrase than acetazolamide. There was again no apparent connection between inhibitory activity* against carbonic anhydrase and oral diuretic activity. Thus the carbonic anhydrase inhibitory activity of 4-chloro-2'-methyl-3-sulphamoylbenzanilide was 0·32 relative to acetazolamide (= 1·0), whilst that of the corresponding 4,2'-dichloro-3-sulphamoylbenzanilide was only 0·04. Both compounds were nevertheless approximately equi-effective oral diuretic agents and more potent than the other compounds listed in Table III.

EXPERIMENTAL

Most of the following examples illustrate methods of preparation used for the products listed in the tables, which also include the analyses.

N-4-*Dimethyl*-3-*sulphamoylbenzamide*. A solution of methyl 4-methyl-3-sulphamoylbenzoate (6·8 g.) in 33 per cent ethanolic methylamine (50 ml.) was kept at room temperature for 6 days when excess of amine was boiled off. The residue was dissolved in water and acidified with hydrochloric acid. The *product* (5·2 g.) had m.p. 219–221° after crystallisation from methanol.

4-*Chloro*-NN-*dimethyl*-3-*sulphamoylbenzamide*. (*a*) 3-*Amino*-4-*chloro*-NN-*dimethylbenzamide*. A mixture of 4-chloro-*NN*-dimethyl-3-nitrobenzamide (34·9 g.) and iron powder (35 g.) in 20 per cent ethanol (300 ml.) containing glacial acetic acid (3 ml.) was heated with stirring under reflux for 5 hr., and was then filtered hot. The oily product which separated was isolated with chloroform and purified by distillation. It (22 g.) had b.p. 174° at 1·0 mm. and m.p. 73–75° (from benzene).

(*b*) 4-*Chloro*-3-*chlorosulphonyl*-NN-*dimethylbenzamide*. A solution of the foregoing amine (17 g.) in 24 per cent hydrochloric acid (200 ml.) was diazotised at 5 to 10° by the addition of a solution of sodium nitrite (7 g.) in water (20 ml.) and the diazo solution added slowly with stirring to a saturated solution of sulphur dioxide in acetic acid (160 ml.) containing cupric chloride dihydrate (8·5 g.). Stirring was continued for 30 min. after the addition was complete. The solution was then diluted with ice-water and the sulphonchloride collected and drained. A portion, crystallised from benzene-light petroleum (b.p. 60–80°) had m.p. 106–108°.

* Determined with Mr. B. G. Overell, M.Sc., and Mrs. S. Ray.

(*c*) A solution of the foregoing sulphonchloride in chloroform (100 ml.) was added with stirring to ammonia solution (170 ml., d = 0·880) and stirring was continued for 30 min. after the addition was complete. Excess of ammonia and chloroform were then boiled off and the residue acidified with hydrochloric acid. The sulphonamide which separated had m.p. 145–146° after crystallisation from water.

4-*Chloro*-N-*isopropyl*-3-*sulphamoylbenzamide.* (*a*) 4-*Chloro*-3-*chlorosulphonylbenzoyl chloride.* A mixture of *p*-chlorobenzoic acid (156 g.), pentachloroethane (120 ml.) and chlorosulphonic acid (266 ml.) was heated under reflux for 4 hr., cooled and poured with stirring on to crushed ice. The solid was collected, washed with ice-cold water, dissolved in 1,2-dichloroethane, washed again with water and the organic layer dried with calcium chloride. The solution so obtained was added to thionyl chloride (140 ml.), dimethylformamide (10 ml.) added as catalyst, and the mixture heated under reflux for 2 hr. Excess of solvent was boiled off and the residual oil distilled at 1·5 mm. to yield the *diacid chloride* (80 per cent yield), b.p. 136–138° and m.p. 42–43° (from ether).

(*b*) 4-*Chloro*-3-*chlorosulphonyl*-N-*isopropylbenzamide.* A solution of the foregoing compound (13·7 g.) in toluene (130 ml.) was cooled to −20° and treated with stirring with a solution of isopropylamine (6·0 g.) in toluene (60 ml.). The mixture was allowed to warm up to 10°, and was then filtered to remove isopropylamine hydrochloride. Dilution of the filtrate with light petroleum furnished the *product* (10·8 g.), m.p. 117–119° after crystallisation from toluene-light petroleum (b.p. 60–80°).

(*c*) A solution of the foregoing sulphonchloride (9 g.) in chloroform (90 ml.) was added with stirring to ammonia solution (90 ml., d = 0·880) and stirring was continued for 30 min. after the addition was complete. Then the excess of chloroform and ammonia was boiled off. The residual *product* (7·6 g.) had m.p. 222–224° after crystallisation from aqueous methanol.

2′-*Methyl*-3-*sulphamoylbenzanilide.* A solution of *m*-sulphamoylbenzoic acid (40 g.) in *o*-toluidine (40 ml.) was heated at reflux temperature, with slow removal of the water formed, for 2 hr. After cooling, the residue was extracted with 10 per cent aqueous potassium hydroxide solution (100 ml.), the extract was diluted to 800 ml., boiled with charcoal and filtered hot. The filtrate was acidified and filtered hot. The residual *product* (39 g.) had m.p. 190–191° after crystallisation from aqueous ethanol.

4-*Chloro*-2′-*methyl*-3-*sulphamoylbenzanilide.* 1. (*a*) 4-*Chloro*-2′-*methyl*-3-*nitrobenzanilide.* A solution of *o*-toluidine (42·8 g.) in chloroform (50 ml.) was added below 15°, with stirring and cooling, to a solution of 4-chloro-3-nitrobenzoyl chloride (39·6 g.) in chloroform (250 ml.). The *product* was collected after a further 15 min.; it had m.p. 151–153° (after crystallisation from benzene). A small second crop of material

was obtained by concentration of the chloroform liquors (total yield, 47 g.).

(*b*) 3-*Amino*-4-*chloro*-2'-*methylbenzanilide*. A mixture of the foregoing nitro-compound (36·2 g.), iron powder (34 g.), water (250 ml.), ethanol (50 ml.) and acetic acid (2·5 ml.) was heated with stirring, under reflux, for 6 hr., and then filtered hot. The residual solid was extracted with boiling ethanol and the extract added to the filtrate. Concentration of the filtrate yielded the amine (24·2 g.), m.p. 160–162° after crystallisation from aqueous ethanol.

(*c*) 4-*Chloro*-3-*chlorosulphonyl*-2'-*methylbenzanilide*. A solution of the foregoing amine (23·8 g.) in 24 per cent hydrochloric acid (200 ml.) was diazotised at 0–5° by the addition of a solution of sodium nitrite (6·3 g.) in water (15 ml.), and the solution added dropwise, with stirring, at 20–25° to a saturated solution of sulphur dioxide in acetic acid (250 ml.) containing cupric chloride dihydrate. Stirring was continued for a further 30 min. and precipitation of the sulphonchloride was completed by dilution with ice-water. It was collected, washed with ice-water and drained. A sample, crystallised from chloroform-light petroleum (b.p. 60–80°), had m.p. 161–163°.

(*d*) A suspension of the foregoing sulphonchloride (40 g.) in chloroform (200 ml.) was added with stirring to aqueous ammonia (400 ml., d = 0·880) and the stirring continued for 1 hr. after the addition was complete. The sulphonamide separated and was collected. It had m.p. 240–242° after crystallisation from aqueous ethanol. The overall yield in the last two stages was 55 per cent.

2. 4-*Chloro*-3-*chlorosulphonyl*-2'-*methylbenzanilide*. (*a*) *o*-Toluidine (107 g.) was added gradually with stirring to a solution of 4-chloro-3-chlorosulphonylbenzoyl chloride (273 g.) in chlorobenzene (1,650 ml.) and the mixture heated under reflux for about 45 min. The solution was cooled slightly and diluted with light petroleum (b.p. 80–100°) until crystallisation began. The *product* (296 g.) had m.p. 162–163° after crystallisation from chlorobenzene-light petroleum (b.p. 60–80°). It was identical with the product described in 1 (*c*).

(*b*) *o*-Toluidine hydrochloride (7·1 g.) was mixed with 4-chloro-3-chlorosulphonylbenzoyl chloride (13·6 g.) in chlorobenzene (200 ml.), and the mixture was heated at reflux temperature for 40 min. The sulphonchloride (14·6_6 g.), isolated by dilution with light petroleum, had m.p. 162–163°.

3. 4-*Chloro*-2'-*methyl*-3-*sulphamoylbenzanilide*. A solution of 4-chloro-3-sulphamoylbenzoic acid (11·8 g.) in *o*-toluidine (20 ml.) was heated in an atmosphere of nitrogen at 190–200° for 1·5 hr., and poured with stirring into 2N hydrochloric acid. The solid was purified by recrystallisation from 50 per cent ethanol to yield the product (7 g.), m.p. 238–239°, which was not depressed on admixture with the material described under 1 (*d*).

4-*Chloro*-3,4′-*disulphamoylbenzanilide.* To a stirred mixture of sulphanilamide (8·6 g.) and anhydrous sodium acetate (4·0 g.) in glacial acetic acid (50 ml.) at 70–80° was slowly added a solution of 4-chloro-3-chlorosulphonylbenzoyl chloride (13·6 g.) in acetic acid (27 ml.). Enough water was added to dissolve sodium chloride and the *sulphonchloride* crystallised on cooling. It was collected, washed with cold water, and added in portions with stirring to ammonia solution (300 ml., d = 0·880). When the addition was complete, excess of ammonia was boiled off; the disulphonamide then separated on cooling. It had m.p. 294–296° after crystallisation from aqueous ethanediol.

2′-*Carboxy*-4-*chloro*-3-*sulphamoylbenzanilide.* (*a*) A mixture of anthranilic acid (6·9 g.) and anhydrous sodium acetate (4·1 g.) in acetic acid (70 ml.) was heated to 80°, treated with a solution of 4-chloro-3-chlorosulphonylbenzoyl chloride (13·6 g.) in acetic acid (20 ml.), and the whole heated to 110° to complete the reaction.The crude *sulphonchloride*, isolated by dilution, was added to ammonia solution (150 ml., d = 0·880) and excess of ammonia was then boiled off. Acidification of the residual liquid yielded the product (10 g.), m.p. 264–266° (decomp.) after crystallisation from aqueous ethanediol.

(*b*) 4-Chloro-2′-methoxycarbonyl-3-sulphamoylbenzanilide (13·8 g.) was dissolved in warm 0·5N sodium hydroxide (200 ml.) and the solution left overnight at room temperature. Acidification furnished the product (12 g.). It had m.p. 264–266° (decomp.) after crystallisation from 30 per cent aqueous dimethylformamide. The m.p. was not depressed on admixture with the compound prepared in (*a*).

4-*Chloro*-2′-*methyl*-3-*methylsulphamoylbenzanilide.* A solution of 4-chloro-3-chlorosulphonyl-2′-methylbenzanilide (11·2 g.) in chloroform (180 ml.) was added with stirring and cooling below 10° to 10 per cent aqueous methylamine solution (100 ml.) and stirring was continued for 30 min. after the addition was complete. The product (8·4 g.) was collected and had m.p. 223–224° after crystallisation from aqueous methanol. Found: C, 53·2; H, 4·3; N, 8·2; S, 9·8. $C_{15}H_{15}ClN_2O_3S$ requires C, 53·2; H, 4·5; N, 8·3; S, 9·5 per cent.

4-*Chloro*-3-*ethylsulphamoyl*-2′-*methylbenzanilide* had m.p. 161–162° after crystallisation from toluene. Found: C, 54·5; H, 4·8; Cl, 9·9; N, 8·1; S, 9·2. $C_{16}H_{17}ClN_2O_3S$ requires C, 54·5; H, 4·9; Cl, 10·1; N, 7·9; S, 9·1 per cent.

4-*Chloro*-3-*dimethylsulphamoyl*-2′-*methylbenzanilide* had m.p. 134–136° after crystallisation from methanol. Found: C, 54·7; H, 4·7; Cl, 10·0; N, 8·1; S, 9·1. $C_{16}H_{17}ClN_2O_3S$ requires C, 54·5; H, 4·9; Cl, 10·1; N, 7·9; S, 9·1 per cent.

3-*Bromo*-2′-*methyl*-4-*sulphamoylbenzanilide.* A solution of 3-bromo-4-sulphamoylbenzoic acid (4 g.) in *o*-toluidine (10 ml.) was heated under reflux in an atmosphere of nitrogen for 2 hr., the mixture cooled somewhat and poured with stirring into dilute hydrochloric acid. The resultant

solid was dissolved in dilute sodium hydroxide solution, brought to pH 10 with acetic acid and filtered hot. The insoluble *product* was washed with hot water. It (2 g.) had m.p. 220–222° after crystallisation from aqueous methanol. Found: C, 45·5; H, 3·7; Br, 21·6; N, 7·3. $C_{14}H_{13}BrN_2O_3S$ requires C, 45·5; H, 3·5; Br, 21·7; N, 7·6 per cent.

3-*Bromo*-2'-*chloro*-4-*sulphamoylbenzanilide* had m.p. 226–228° after crystallisation from aqueous ethanol. Found: C, 40·4; H, 2·9; N, 6·8; S, 7·9. $C_{13}H_{10}BrClN_2O_3S$ requires C, 40·1; H, 2·6; N, 7·2; S, 8·2 per cent.

3-*Chloro*-2'-*methyl*-4-*sulphamoylbenzanilide*. A solution of 3-chloro-4-sulphamoylbenzoic acid (10 g.) in *o*-toluidine (45 ml.) was distilled over 1 hr. under nitrogen to remove most of the base, last traces of which were distilled off at reduced pressure. The residual solid was dissolved in 0·5N sodium hydroxide solution (500 ml.), heated to near boiling and filtered hot. The insoluble *product* (4·1 g.) had m.p. 218–220° after crystallisation from aqueous ethanol. Found: C, 52·1; H, 4·3; N, 9·1; S, 10·1. $C_{14}H_{13}ClN_2O_3S$ requires C, 51·8; H, 4·0; N, 8·6; S, 9·9 per cent.

References

Barr, C. R., Salminen, I. F. and Weissberger, A. (1951). *J. Amer. chem. Soc.*, **73**, 4131–4133.
Jackman, G. B., Petrow, V., Stephenson, O. and Wild, A. M. (1962). *J. Pharm. Pharmacol.*, **14**, 679–686.
Montagne, P. J. (1900). *Rec. Trav. chim.*, **19**, 46–78.
Petrow, V., Stephenson, O. and Wild, A. M. (1960). *J. Pharm. Pharmacol.*, **12**, 705–719.
Smiles, S. and Stewart, J. (1921). *J. chem. Soc.*, **119**, 1792–1798.
Ullmann, H. M. (1894). *Amer. chem. J.*, **16**, 530–543.
Wegscheider, R. and Furcht, M. (1902). *Monatsh.*, **23**, 1093–1146.

A NOTE ON THE EFFECT OF HAMYCIN ON ELECTROLYTES IN THE FROG HEART

By H. R. K. Arora* and V. Arora†

*From the *Department of Pharmacology, Maulana Azad Medical College, and †Department of Pharmacology, All India Institute of Medical Sciences, New Delhi, India*

Received September 13, 1962

Treatment of frog heart with hamycin, an antifungal antibiotic, effected an increase in the potassium content of perfusion fluid. Changes in sodium content were not observed.

Arora (1962) first reported the presence of cardiotonic activity in hamycin, a new antifungal antibiotic. An investigation was thereafter undertaken to study the effects of hamycin on the sodium and potassium in frog heart.

Experimental and Results

Hamycin solution was prepared by adding 0·1 g. of the powder to 10 ml. of propylene glycol at 85°. A 0·6 per cent solution (w/v) was obtained, the rest remaining insoluble. The soluble fraction was dissolved in amphibian Ringer-Lock solution to give a concentration of 10^{-6} g./ml.

Rana temporaria of either sex were used to prepare Straub hearts. One ml. of Ringer-Locke solution containing the same amount of propylene glycol as in the hamycin solution, was put into the cannula and left for 5 min. The fluid was then removed and kept as control for electrolyte estimation. One ml. of hamycin solution was then put into the cannula and left until systolic arrest was complete. This fluid was also collected and retained for electrolyte estimation. Similar experiments were made with tincture of digitalis, 25 ml./litre. In 3 control experiments, Ringer-Locke solution containing only propylene glycol was left in contact for 15 min.

After the systolic arrest was complete, the ventricle was removed, blotted on filter paper and quickly weighed on a torsion balance. It was then dried overnight at 115° and the dry weight recorded.

Sodium and potassium estimations were made on a Perkin Elmer flame photometer.

A concentration of 10^{-6} g./ml. of hamycin or 25 ml./litre of tincture digitalis induced systolic arrest in the Straub-Feuhner preparation in 5 to 10 min. Control preparations beat normally over this period.

Hamycin treatment resulted in an increase in the potassium content of the perfusion fluid (Table I). The change, when expressed in terms of tissue water amounted to 9·2 m-equiv./litre $\pm$1·38 s.e. A similar increase was noted with tincture of digitalis (Table I). There was no change in the electrolyte content of perfusion fluid in control experiments.

No definite change in the sodium content was observable, either with tincture of digitalis or hamycin (Table I) and no alteration in the tissue water content could be detected as a result of treatment with either drug.

Discussion

Hajdu and Leonard (1959) in their review of the mechanism of action of cardiotonic drugs suggested that the cardiotonic action of digitalis might be due to a loss of tissue potassium, a slight gain in tissue sodium and some loss of tissue water but without any alteration in the total concentration of intracellular monovalent cations. Such effects would theoretically result in an increase in potassium and a decrease in sodium content of the perfusion fluid. Hamycin treatment of the frog heart resulted in a definite increase in the potassium content of the perfusion

TABLE I

THE EFFECTS OF HAMYCIN AND DIGITALIS ON THE ELECTROLYTE CONTENT OF PERFUSION FLUID IN THE STRAUB-HEART PREPARATION

Drug concentration	Heart weight mg.		Tissue water mg.	Water per cent	Mean water ±s.e. per cent	Change in electrolyte content of fluid (m. equiv./litre)		Change in potassium m-equiv./litre of tissue water	Mean change in potassium ±s.e.
	Wet	Dry				Potassium	Sodium		
Hamycin 10^{-6} g./ml.	159	24·75	134·25	84·4		+1·275	nil	9·5	
Hamycin ,,	200	31·15	168·85	84·5		+0·675	nil	4·0	
Hamycin ,,	131	20·53	110·47	84·3	84·2	+1·05	nil	9·5	9·2
Hamycin ,,	80	12·2	67·8	84·7	±0·23	+0·825	nil	12·2	±1·38
Hamycin ,,	102	17·1	84·9	83·3		+0·900	−2·41	10·6	
Tr. digitalis 25 ml./l.	102	17·66	84·34	82·7		+0·900	nil	10·6	
Tr. digitalis ,,	146	28·18	119·82	82·1	82·9	+0·600	nil	5·0	7·0
Tr. digitalis ,,	83	13·32	69·68	83·9	±0·53	+0·375	nil	5·4	±1·86
Control	103	18·99	84·01	81·5		nil	nil	nil	
Control	100	15·35	84·65	84·65	82·65	nil	nil	nil	
Control	85	15·5	69·50	81·8	±1·00	nil	nil	nil	

fluid, an effect known for digitalis and also confirmed in the present study. The change was particularly marked when calculated in terms of tissue water. Although the results do not represent quantitative exchanges between intracellular and extracellular fluids, there can be little doubt that the extra potassium in the perfusion fluid must have been derived from the cells. The inability to show any changes in sodium content of the perfusion fluid was due to the fact that changes in tissue sodium content are known to be slight (Hadju and Leonard, 1959) and are therefore not likely to be detected in the perfusion fluid which itself has a high sodium content.

The effect of hamycin on potassium thus further confirms its digitalis like activity.

Acknowledgements. The authors thank the Director, All India Institute of Medical Sciences, New Delhi, India, and the Principal, Maulana Azad Medical College, New Delhi, India, for facilities. Hamycin was obtained through the courtesy of Dr. B. B. Gokhalay, Poona, India.

References

Arora, H. R. K. (1962). *J. Pharm. Pharmacol.*, **14**, 320.
Hajdu, S. and Leonard, E. (1959). *Pharmacol. Rev.*, **11**, 173–209.

LETTERS TO THE EDITOR

Binding of Quinidine to Myocardial Cellular Components

SIR,—The distribution of quinidine in tissues has been studied by Hiatt and Quinn (1945) and Kelsey, Oldham and Geiling (1945). We are concerned with the intracellular localisation of quinidine in the cardiac tissue of the dog.

Sixteen healthy mongrel dogs, of both sexes, weighing 7 to 15 kg. were used. Quinidine sulphate (Merck), dissolved in distilled water with a few drops of 0·1 N H_2SO_4 and neutralised to pH 7·0, was administered intravenously in a dose of 15 mg./kg. weight. Fifteen min. after the injection, samples of tissue from the left ventricle were excised under ether anaesthesia (our preliminary experiments demonstrated that the left ventricle attained the highest quinidine concentration). After removing fibrous tags and blood and thoroughly mincing with scissors, the tissue was homogenised in a chilled Monel-metal Waring blendor with cold isotonic sucrose solution for exactly 2 min., the homogenate was passed through muslin to remove connective tissue and cell debris and finally made to volume as a 10 per cent homogenate. Ten ml. of the homogenate was then differentially centrifuged at 4° in an International Refrigerated Centrifuge, Model PR-2, and various components of the cell (nuclei, sarcosomes and supernatant fraction) were isolated by adopting the technique of Schneider and Hogeboom (1950) for liver. Microsomes, however, could not be separated in this way. For *in vitro* studies, 10 ml. of the 10 per cent homogenate was incubated with 20 μg. quinidine for 15 min. at 37°, followed by separation into the different fractions. Quinidine was estimated in duplicate samples on a Klett Summerson fluorimeter by the method of Kelsey and Geiling (1942). Added quinidine gave 88–95 per cent recovery.

TABLE I

INTRACELLULAR LOCALIZATION OF QUINIDINE IN LEFT VENTRICLE OF DOG

	Homogenate	Nuclei		Sarcosomes		Supernatant*	
	Units	Units	Per cent	Units	Per cent	Units	Per cent
In vivo (10) s.e.	24·91 ±1·38	12·90 ±0·93	52	1·87 ±0·15	8	8·14 ±0·26	33
In vitro (6) s.e.	18·30 ±0·29	9·00 ±0·26	49	0·83 ±0·11	5	7·15 ±0·63	39

Units = μg. quinidine per g. equivalent tissue. * Fraction containing microsomes.
Number of animals in parentheses.

The intracellular localization of quinidine in the left ventricle (Table I) was significantly higher in the nuclear fraction than in the sarcosomal and supernatant fractions. The percentage distribution of quinidine in nuclei, sarcosomes and supernatant in the *in vivo* studies was respectively 52, 8 and 33 and 49, 5 and 39 in the *in vitro* studies. Further investigations are in progress in this laboratory. Financial assistance from the Indian Council of Medical Research is gratefully acknowledged.

Department of Pharmacology,
All-India Institute of Medical
Sciences, New Delhi-16, India.
December 4, 1962

R. B. ARORA
J. N. SHARMA
T. K. TARAK
Y. R. SAXENA

REFERENCES

Hiatt, E. P. and Quinn, G. P. (1945). *J. Pharmacol.*, **83**, 101–105.
Kelsey, F. E., Oldham, F. K. and Geiling, E. M. K. (1945). *Ibid.*, **85**, 170–175.
Kelsey, F. E. and Geiling, E. M. K. (1942). *Ibid.*, **75**, 183–186.
Schneider, W. C. and Hogeboom, G. H. (1950). *J. biol. Chem.*, **183**, 123–128.

Influence of Guanethidine on the Catecholamine Depleting Effects of Tyramine in the Rat Heart

SIR,—Tyramine showed no pressor action in animals whose noradrenaline stores were depleted with reserpine (Carlsson and others, 1957), but its actions were re-established by prolonged infusion with noradrenaline (Burn and Rand, 1958). It was predominately these facts which led the same authors to advance the hypothesis that the actions of tyramine are mediated through the release of noradrenaline. Studies carried out in this laboratory as well as by Potter and others (1962) have provided direct evidence for this hypothesis in the intact animal.

Since guanethidine has been shown to antagonise the pressor action of tyramine (Maxwell, Plummer, Povalski and Schneider, 1960) and tyramine exerts its effects by liberating noradrenaline, guanethidine would be expected to prevent the depleting action of tyramine. This possibility was examined in the following experiment. Male rats (Holtzman Strain), weighing 200 to 225 g. were given guanethidine sulphate 5 mg./kg. intravenously 20 min. before the intramuscular injection of tyramine hydrochloride (20 mg./kg.). 30 min. after tyramine administration the animals were killed by decapitation. The concentrations of catecholamines in the ventricular myocardium were determined by the trihydroxyindole fluorimetric procedure of Shore and Olin (1959) and are expressed as μg. of noradrenaline per g. of fresh tissue.

TABLE I

EFFECT OF GUANETHIDINE ON THE CATECHOLAMINE DEPLETING EFFECT OF TYRAMINE IN THE RAT HEART

Treatment	Number of animals	Catecholamine concentration μg./g. of fresh tissue. Mean ± s.e.
None	18	1·06 ± 0·03
Tyramine	11	0·70 ± 0·02
Guanethidine	7	0·95 ± 0·05
Guanethidine + tyramine	18	0·67 ± 0·03

The results, summarised in Table I, show that guanethidine had no effect on the depleting effects of tyramine in the rat heart. The observation of Lindmer and Muscholl (1961) that guanethidine significantly reduces the tyramine-induced release in the isolated, perfused rabbit heart appears to be unrelated to its pharmacological action in the intact animal.

B. BHAGAT*

Department of Pharmacology and Toxicology,
The University of Wisconsin,
Madison, Wisconsin, U.S.A.
December 7, 1962

REFERENCES

Burn, J. H. and Rand, M. J. (1958). *J. Physiol. (Lond.)*, **144**, 314–336.
Carlsson, A., Rosengren, E., Bertler, A. and Nilsson, J. (1957). In *Psychotropic Drugs*, p. 363, Elsevier Publishing Co., Amsterdam.
Lindmar, R. and Muscholl, E. (1961). *Arch. exp. Path. Pharmak*, **242**, 224.
Maxwell, R. A., Plummer, A. J., Povalski, H. and Schneider, F. (1960). *J. Pharmacol.*, **129**, 24–30.
Potter, L. T., Axelrod, J., Kopin, I. J. (1962). *Biochem. Pharmacol.*, **11**, 254–256.
Shore, P. A. and Olin, J. S. (1959). *J. Pharmacol.*, **122**, 295–300.

* Present address: Department of Pharmacology, University of Minnesota Medical School, Minneapolis 14, Minnesota, U.S.A.

The Action of Anti-inflammatory Drugs on the Permeability of Mesenteric Mesothelium to Plasma Protein

SIR,—Mesenteric mesothelium bears a close morphological resemblance to the endothelium of blood vessels and it was of interest to discover whether drugs which reduced vascular permeability would also reduce the permeability of the mesentery to plasma protein. A method has been devised which enables the permeability of the rat mesentery to plasma protein to be measured *in vitro*. A large rat is killed by a blow on the head and a section of small intestinal mesentery is selected which is free from major blood vessels and fat deposits. The mesentery is gently draped over an open end of a 12 mm. diameter glass tube and secured around the rim with a cotton ligature. The piece of mesentery occluding the end of the glass tube is then cut out from the abdomen. In this way it is possible to rapidly complete the preparation without touching the surface of the delicate mesentery with either the fingers or instruments. The mesenteric diaphram is then set up in a 100 ml. beaker containing 70 ml. of Locke's solution, whilst inside the glass tube is placed 2·5 ml. of solution containing 0·5 per cent bovine plasma albumin, 0·5 per cent azovan blue, and 0·9 per cent sodium chloride. Care is taken to maintain the levels of fluid inside and outside the tube the same at all times. The Locke's solution is kept mixed by means of a slowly rotating magnetic stirrer, and the temperature maintained at 26–28°.

Albumin-bound azovan blue slowly diffuses through the mesentery into the Locke's solution, 1 ml. samples of which are withdrawn at 10 min. intervals and their optical extinction measured. Each time a sample is taken 1 ml. of fresh Locke's solution is added to keep the volume constant. Samples are collected in this way for 90 min. and then the substance to be tested is added to the bath as a neutral (pH 7·4) solution, samples being collected for a further 90 min. The rate of entry of dye (G) is calculated by the method of least squares both for the period before, and the period after the addition of the substance under test. The ratio of the rate of diffusion before (G_1) and after (G_2) addition of the drug is a measure of the effect of the drug on the permeability of the mesentery to plasma protein.

TABLE I

ACTION OF DRUGS ON THE PERMEABILITY OF MESENTERY TO PLASMA-PROTEIN

Drug	G_2/G_1 per cent
Control (sodium chloride) ..	91
2,6-Dihydroxybenzoic acid ..	93
m-Hydroxybenzoic acid	86
Salicylic acid	75*
Thymoxyacetic acid	62*

Final concentration of each substance in the bath was 7·5mM.

* Indicates that there is a significant ($P < 0.05$) difference between G_1 and G_2.

Table I shows the effects of various substances. Thymoxyacetic acid was the most active of those tested and this is in agreement with the considerable activity of the substance as an anti-inflammatory agent (Northover and Verghese 1962). Among hydroxybenzoates 3 compounds were tested, salicylate being active, *m*-hydroxybenzoate only slightly active, and 2,6-dihydroxybenzoate inactive. This again is in agreement with the anti-inflammatory action of the compounds.

It would seen that the rat mesentery offers a useful *in vitro* system on which the effect of permeability-reducing drugs can be investigated and these preliminary observations suggest that it behaves in some ways like the endothelium of blood vessels.

B. J. NORTHOVER

Department of Pharmacy,
Christian Medical College, Vellore,
South India.
December 7, 1962

REFERENCE

Northover, B. J. and Verghese, J. (1962). *J. Pharm. Pharmacol.*, **14**, 615–616.

Effect of Pyrogallol on Acute Learning in Rats

SIR,—Pyrogallol causes an increase in the contents of adrenaline and noradrenaline of the brain of mice (Izquierdo, J. A. and Biscardi, 1961) and rats (Izquierdo, J. A., Juorio and Dezza, unpublished) presumably by inhibition of their *O*-methylation. It seemed to us, therefore, a useful tool for a preliminary approach to the study of adrenergic mechanisms in learning.

At a dose of 200 mg.kg. i.p., in rats, it promoted habituation of investigatory-orienting and of arrest unconditioned reflexes to a buzzer to which there normally was none. The startle reflex was unaffected. The buzzer had a duration of 3 sec. and was presented 50 consecutive times (intervals between buzzes: 30–90 sec.) to 26 animals, 19 of which received pyrogallol and 7 water, 3 min before testing.

In other groups of rats, after 20 "control" presentations of this buzzer alone, it was paired with a shock (0·5 sec. of 150/sec. 100 V × 0·1 msec. rectangular pulses) delivered 1–2 sec. after it to a metallic grid on the floor of the training box. An instrumental response (lifting of one or both forepaws) appeared after 5–15 pairings, and reached a stable level of 50–90 per cent per block of 10 trials after 40–50 trials. The initial rate of appearance of the conditioned reflex was significantly lower in 9 rats to which pyrogallol was given 3 min. before the session began, as compared with 7 water-treated ones. In 8 other rats in which pyrogallol was given later during the reinforcement stage, when the instrumental response was already stabilised, its performance was unaffected.

In 7 rats, pyrogallol was injected 3 min. before beginning an extinction of the conditioned reflex; the rate of the extinction was significantly higher than in 7 other water-treated rats.

Pyrogallol is known to produce a slight increase in blood pressure which is counteracted by phentolamine (Izquierdo, 1962), and an increase in duodenal motility which is blocked by atropine (Izquierdo and Izquierdo, 1961; Izquierdo, 1962), both effects lasting 15–40 min. Neither phentolamine (10 mg./kg., 7 rats) nor atropine (1 mg./kg., 7 rats) given i.p. 1 min. before pyrogallol, modified the effect of the latter on extinction. Phentolamine alone (7 rats) had an effect not different from that of water, but atropine (7 rats) increased the extinction to a level not significantly different from that attained either with pyrogallol alone or with both drugs in combination.

Thus, any reflex influence of blood pressure or increase of duodenal motility by pyrogallol on extinction can be disregarded, and an effect on brain catecholamines is left as the most likely mechanism. But the action of atropine on extinction suggests, nevertheless, some interaction (other than duodenal) between both drugs.

Habituation and extinction, and possibly sleep, belong to the same category of phenomena, those of "internal inhibition." This is a process opposed by reinforcement, for it tends not to appear in its presence (Pavlov, 1960). This might explain the fact that in our rats a conditioned response was unaffected by pyrogallol during the reinforcement stage, whereas pyrogallol clearly enhanced internal inhibitory processes in situations where reinforcement was absent, like habituation, or extinction.

The decreased rate of establishment of a conditioned reflex, in view of the results on habituation, may be due to the fact that the "inhibitory property" of the conditioned stimulus (Konorski, 1948) was increased by pyrogallol.

In no experiment did our rats show any motor disturbance nor any apparent neurological symptom. The response to shock itself was obviously unmodified by pyrogallol.

Our data on enhanced internal inhibition by pyrogallol, if in fact due to the increase in cerebral catecholamines, may be in agreement with those that ascribe a "central inhibitory," sleep-inducing property to centrally active catecholamines (Bass, 1914; Domer and Feldberg, 1960). Attention is obviously drawn towards those diencephalic and mesencephalic structures which are normally rich in these transmitters (Vogt, 1954).

IVAN IZQUIERDO*
ALICIA B. MERLO

Catedra de Farmacologia Experimental,
Facultad de Farmacia y Bioquimica,
Junin 956. Buenos-Aires. Argentina.
December 6, 1962

REFERENCES

Bass, A. (1914). *Z. ges. Neurol. Psychiat.*, **26**, 600–602.
Domer, F. R. and Feldberg, W. (1960). *Ciba Symp. on Adrenergic Mechanisms.* Editors: Vane, J. R., Wolstenholme, G. E. W., O'Connor, M, Boston: Little, Brown & Co.
Izquierdo, I. (1962). Doctor's Thesis. University of Buenos-Aires.
Izquierdo, I. and Izquierdo, J. A. (1961). *J. Pharm. Pharmacol.*, **13**, 743–746.
Izquierdo, J. A. and Biscardi, A. (1961). *Rev. Soc. argent. Biol.* (in press).
Konorski, J. (1948). *Conditioned Reflexes and Neuron Organization.* Cambridge: Univ. Press.
Pavlov, I. P. (1960). *Conditioned Reflexes.* Dover, New York.
Vogt, M. (1954). *J. Physiol. (Lond.)*, **123**, 451–481.

* Present address: Dept. Anatomy, U.C.L.A. Med. Center, Los Angeles 24, Calif., U.S.A.

Lecithin-cholesterol Sols

SIR,—Further studies have been made of the clear lecithin-cholesterol sols formed by ultrasonic irradiation of coarse dispersions (Saunders, Perrin and Gammack, 1962). Electron micrographs of the residues obtained when the sols are dried down with sodium phosphotungstate as a negative stain, indicate that the lecithin sols alone give round particles many of which have a mean diameter in the range 100 to 200 Å, while the lecithin-cholesterol sols show many membrane-like structures of thickness 40 to 50 Å. A 1 : 1 molar ratio is the maximum cholesterol : lecithin ratio which gives stable dispersions.

The formation of interfacial membranes from concentrated lecithin-cholesterol sols has been examined. Since a cell membrane is probably formed by a precipitation reaction between the cell contents containing a high concentration of lipid and the environmental fluid, a film precipitated at an interface between a lecithin-cholesterol sol and another aqueous solution should give a realistic model of a natural membrane.

The clear lecithin-cholesterol sols are not easily precipitated, but on mixing with a bovine plasma albumin sol they give a flocculent precipitate of lipoprotein; the rate of flocculation is increased by the presence of calcium salts. The possibility of forming this insoluble complex as a film between the lipid sol and an albumin sol has been examined. The lipid sol (10 per cent lecithin, 5 per cent cholesterol) was placed above the albumin sol (10 per cent albumin) to give a sharp boundary. If the albumin sol contained calcium chloride (0·001—0·01 N) a membrane possessing considerable elasticity developed at the interface after about 3 hr. The rate of formation of the membrane could be increased by adding calcium chloride to the lipid sol; 0·01 N calcium chloride did not cause any increase in the turbidity of the sol.

A capillary diffusion apparatus similar to that described by Saunders (1960) has been used to attempt to determine the permeability of this interfacial film to salts. Albumin and lecithin-cholesterol sols were treated with mixed ion-exchange resins until their electrical conductivities were negligible. Calcium chloride solution was then added to both sols to give them equal conductivities the final concentration of this salt in each sol being about 0·01 N. In addition, sodium or potassium chloride was added to the albumin sol only, to give a concentration of 0·01 N. Thus the standard system at 24° was:

10 per cent albumin +	10 per cent lecithin +
0·01 N NaCl (or KCl) and $CaCl_2$	5 per cent cholesterol + 0·01 N $CaCl_2$

An interface between the two sols was formed in the capillary 3 mm. above the conductivity electrodes and the rate of diffusion of NaCl or KCl was followed by the conductivity change. With more concentrated sols very low diffusion rates were found, the rate of diffusion of KCl being significantly greater than that of NaCl. Some results are shown below.

D is the diffusion coefficient in $cm.^2\ sec.^{-1}$.

(1) NaCl, $D = 2 \times 10^{-7}$; D into water 150×10^{-7}.

(2) KCl, $D = 15 \times 10^{-7}$; D into water 165×10^{-7}.

(3) NaCl, no $CaCl_2$ in the system, $D = 41 \times 10^{-7}$.

(4) NaCl, 2 per cent lysolecithin in lipid sol, $D = 15 \times 10^{-7}$.

(5) NaCl, colloid components at half concentrations of standard, $D = 40 \times 10^{-7}$.

In experiments (3), (4) and (5) no clear interfacial films were formed.

Acknowledgments are made to A. D. Bangham and R. Horne for the electron micrographs and to D. Russell for his help in preparing the sols.

School of Pharmacy,
University of London,
29–39 Brunswick Square,
London, W.C.1.

L. SAUNDERS

January 1, 1963

REFERENCES

Saunders, L. (1960). *J. Pharm. Pharmacol.*, **12**, 253*T*-256*T*.
Saunders, L., Perrin, J. and Gammack, D. (1962.) *Ibid.*, **14**, 567-572.

PRINTED BY W. HEFFER & SONS LTD., CAMBRIDGE, ENGLAND.