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Research Papers

Infrared identification of pharmaceutically important steroids with particular reference to the occurrence of polymorphism

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The infrared absorption spectra of steroids, when compared with the spectra of Authentic Specimens, provide a simple and complete means of identification, provided that the effects of polymorphism are precluded. Of 35 substances examined, 16 showed no evidence of polymorphism and a further twelve were sufficiently soluble to be examined in solution. Specific solvent treatments, details of which are given, may be necessary with the remaining seven substances if the spectra of the sample and of the Authentic Specimen are not identical when first examined.

THE establishment of a collection of Authentic Specimens for use with certain tests of the British Pharmacopoeia and the British Pharmaceutical Codex prompted an investigation into the incidence of polymorphism in a number of pharmaceutically important steroids. Thirty-four of the substances examined were required for the preparation of infrared spectra to be used for comparison purposes in qualitative identification tests. The 35th substance, digitoxin, has not been issued as an Authentic Specimen but was included for comparison with digoxin. It was therefore necessary to establish conditions whereby different forms of a substance, should they exist, might be converted to a single form thus eliminating differences in solid-state infrared spectra.

Polymorphism in steroids has been known for at least 30 years and differences in infrared spectra of different forms have been reported (e.g. Dickson, Page & Rogers, 1955; Smakula, Gori & Wotiz, 1957; Callow & Kennard, 1961). Changes in spectrum due to grinding with potassium bromide have also been reported to occur with steroids (Roberts 1957; Hayden & Sammul, 1960); such changes have also been reported with other compounds containing hydroxyl groups (Barker, Bourne, Neely & Whiffen, 1954; Farmer, 1957). In some instances changes in spectrum were ascribed to conversion of a crystalline form into an amorphous form or into a second crystalline form (Baker, 1957). Changes in crystalline form and thus in the absorption spectrum might also be induced by the solvent extraction methods used for the isolation of steroids from pharmaceutical preparations before infra-red examination.

Experimental

MATERIALS

Chloroform, acetone and ethanol used for solvent treatments were of B.P. quality. Samples were prepared for infrared examination using

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liquid paraffin B.P., potassium chloride A.R. or potassium bromide (spectroscopic grade: E. Merck A. G., Darmstadt).

Except where otherwise stated, the steroid samples examined were Authentic Specimens provided by the British Pharmacopoeia and the British Pharmaceutical Codex, and the names used are those under which these specimens are issued.

The chloroform contains up to 2% of ethanol, but no evidence was found to suggest that this affected the crystalline state of substances recovered from chloroform.

SOLVENT TREATMENTS

Each sample was subjected to the following solvent treatments: separate portions were dissolved in minimum amounts of chloroform, of acetone and of ethanol and the solutions evaporated to dryness on a water-bath. Further portions were dissolved in chloroform and also in acetone in an agate mortar and the solvent allowed to evaporate in a current of air at room temperature. Where necessary the last traces of solvent were removed with a jet of air. These solvent treatments will be referred to hereafter as hot chloroform, hot acetone, hot ethanol, cold chloroform and cold acetone. In certain instances, noted below, additional solvents were used. Digoxin, hydrocortisone sodium succinate and prednisolone sodium phosphate were insoluble in chloroform and acetone, and could only be recrystallised from ethanol (50% aqueous ethanol in the last case).

INFRARED ABSORPTION SPECTRA*

Spectra of all the steroids, before and after solvent treatment, were obtained in the solid phase using either a mull in liquid paraffin or an alkali halide disc. Where possible, spectra were also recorded from solutions in chloroform. The procedures used were as recommended in Appendix IV. I. of the British Pharmacopoeia, 1963. Materials for examination as mulls were ground lightly by hand in a mortar before adding liquid paraffin; those used for disc preparation were ground with potassium bromide or potassium chloride for 2 min in a ball mill to ensure thorough mixing before pressing.

All the spectra were obtained by one of us (R.J.M.) using a Grubb Parsons GS 2 grating spectrometer. A Unicam SP 200 spectrometer with sodium chloride prism was used (by C.A.J.) to confirm many of these independently, and also to examine additional samples of many of the substances.

The presence of discrete polymorphic forms was confirmed by means of powder diffraction patterns recorded on a Metropolitan-Vickers Raymax 100 instrument using a 9 cm camera and cobalt K α-radiation.

Results and discussion

Table 1 lists the substances examined and shows those found to exist in more than one solid form and those sufficiently soluble in chloroform

*Throughout the paper the use of the word "spectrum" refers to the infrared absorption spectrum.

TABLE 1. INCIDENCE OF POLYMORPHISM IN THE 35 SUBSTANCES EXAMINED

Substance	No. of solid forms identified	Solution Spectrum possible
Betamethasone†	2	No
Cortisone acetate†	7	Yes
Deoxycortone acetate	1	No
Deoxycortone trimethylacetate	1	No
Dexamethasone	1	No
Dexamethasone acetate†	4	Yes
Digitoxin†	1	No
Digoxin	1*	No
Dimethisterone	2	Yes
Ethinyloestradiol†	3	Yes
Ethisterone	1	No
Fludrocortisone acetate†	4	No
Fluocinolone acetonide†	2	Yes
Fluoxymesterone	1	No
Hydrocortisone	2	No
Hydrocortisone acetate	1	No
Hydrocortisone hydrogen succinate	1	No
Hydrocortisone sodium succinate	1	No
Methylprednisolone	2	No
Methyltestosterone	1	Yes
Norethandrolone	2	Yes
Norethisterone	1	No
Norethynodrel	2	Yes
Prednisolone	2	No
Prednisolone acetate	1	No
Prednisolone sodium phosphate	1	No
Prednisolone trimethylacetate	3	Yes
Prednisone	2	No
Prednisone acetate	2	Yes
Progesterone	2	Yes
Spironolactone†	3	Yes
Testosterone	3	Yes
Testosterone propionate	1	Yes
Triamcinolone†	2	No
Triamcinolone acetonide†	1	No

* A possible second form differed only in one band.
† Differences observed between halide disc and paraffin mull spectra.

for spectra to be obtained in solution. The path length is limited by solvent absorption and in our opinion should not exceed a nominal 0·2 mm; the minimum solubility requirement was therefore about 3%. Table 1 also indicates those compounds in which differences were observed between spectra from a potassium halide disc and those from a liquid paraffin mull of the sample as received; for the most part, the other forms were examined only as mulls.

The publication of reference spectra of steroids is of limited value where several different spectra are possible. Where only one form has been detected however, its spectrum may be regarded as a reliable means of identification, though the possibility of other forms occurring must not be overlooked. Spectra for seven of the steroids examined, and for which we have detected only one solid form, have not previously appeared in readily available literature, and these are reproduced in Figs 1 and 2. The substances exhibiting polymorphism are considered individually below.

Betamethasone. Material recovered from chloroform solution gave the same spectrum as the original form; evaporation of ethanol, acetone and methanol solutions yielded increasing proportions of a second form, but this was not obtained in a pure state. The X-ray powder diffraction pattern of the mixture obtained from methanol solutions showed only lines due to the original material so the second form is presumably

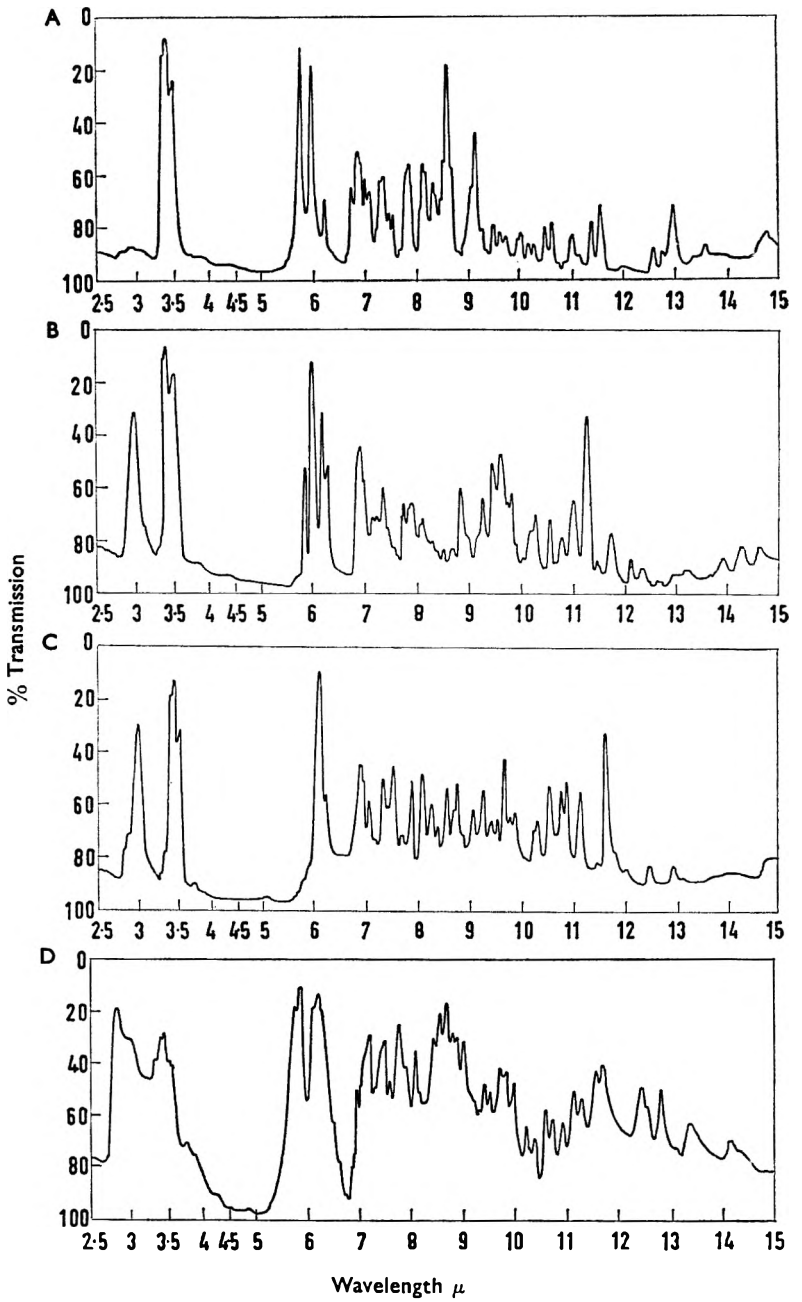

FIG. 1. A. Infrared spectrum of deoxycortone trimethylacetate (liquid paraffin mull). B. Infrared spectrum of dexamethasone (liquid paraffin mull). C. Infrared spectrum of fluoxymesterone (liquid paraffin mull). D. Infrared spectrum of hydrocortisone hydrogen succinate (potassium bromide disc).

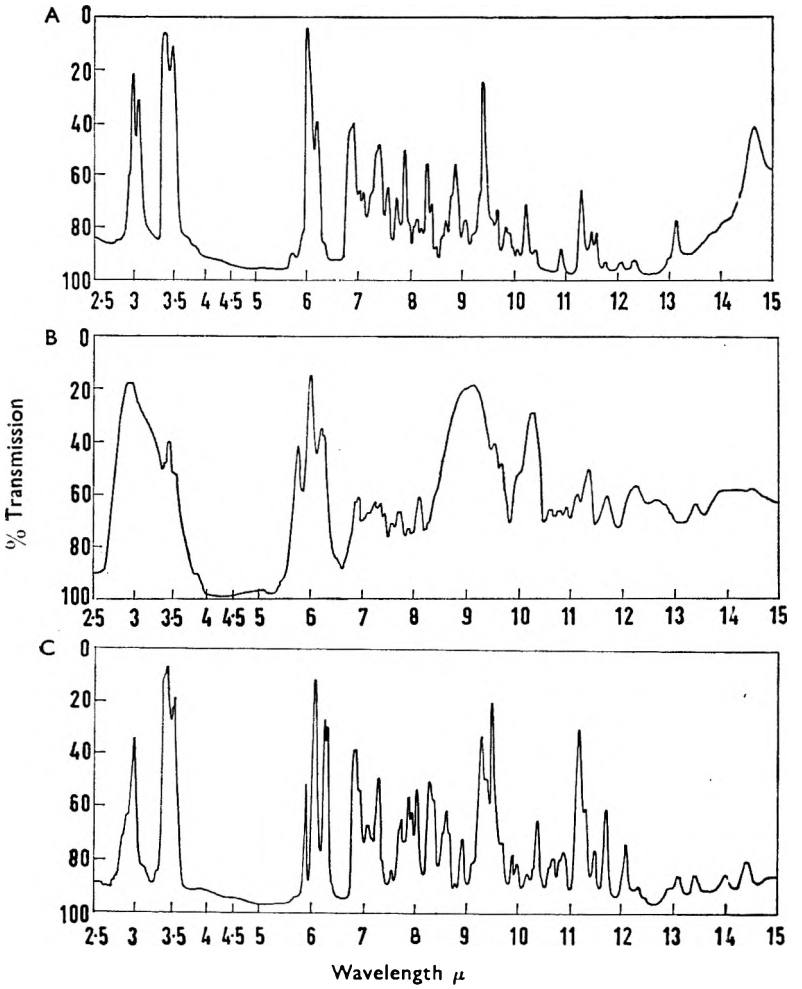

FIG. 2. A. Infrared spectrum of norethisterone (liquid paraffin mull). B. Infrared spectrum of prednisolone sodium phosphate (potassium bromide disc). C. Infrared spectrum of triamcinolone acetonide (liquid paraffin mull).

amorphous. A potassium bromide disc prepared from the original material also showed the presence of this second form.

Cortisone acetate. Callow & Kennard (1961) published X-ray diffraction patterns and partial infrared spectra of five forms. The complete spectrum of form II was published as this form was said to be reproducibly obtained by evaporation of a chloroform solution to dryness. In the present work, evaporation of chloroform solution in a beaker on a water bath gave form III, evaporation in a mortar at room temperature gave form II, and evaporation in a beaker at room temperature gave a mixture of both these forms. Ethanol, hot or cold, gave mixtures of forms I and IV, methanol gave mixtures of forms II and V, and acetone under various

conditions produced forms I, II, III, IV and a new form not described by Callow & Kennard. A mixture of the new form with form I, obtained by evaporation of acetone solution at room temperature, gave an X-ray powder diffraction pattern similar to that recorded by Beher, Parsons & Baker (1955). Many of these mixtures could not be identified solely from their infrared spectra, and X-ray patterns were used to provide confirmation. A seventh form was recovered from tetrahydrofuran solution. Of the specimens examined, the B.P. Authentic Specimen was received as form II; the U.S.P. Reference Standard and the W.H.O. Authentic Chemical Substance were both form III.

Published spectra from other sources show considerable variation: Neudert & Röpke (1957) give form II, agreeing with the spectrum published by Callow & Kennard, while Tarpley Yudis, Manowitz, Horrigan & Weiss (1954) give form I; Hayden, Sammul, Selzer & Carol (1962) who say their material was obtained by evaporation of ethanol solution, give form I but with a peak at 868 cm^{-1} apparently due to form IV; a similar spectrum is included in the Sadtler Pharmaceutical collection; and Meda (1958) shows a spectrum between 1600 and 1800 cm^{-1} which is probably a mixture of forms II and III, but might be a mixture of forms I and IV.

It is clear from this confusion that when any doubt exists about the authenticity of a sample of cortisone acetate it is advisable to record the spectrum in solution.

Dexamethasone acetate. Four forms have been distinguished one of which apparently contains chloroform. None of the solvent treatments used consistently gave a single form, though the material obtained from hot acetone closely resembled the original sample. Solubility in chloroform is only about 3% but this is sufficient to give a characteristic spectrum in a 0·2 mm cell.

Digitoxin. Hayden & others (1962) reported that a potassium bromide disc containing digitoxin showed marked changes in spectrum on heating. This statement has been confirmed, but the heated disc was much more opaque and attenuation of the reference beam was necessary to obtain a good spectrum. A liquid paraffin mull gave a spectrum similar to that of the heated disc and this technique seems preferable; Bell (1960) obtained a comparable spectrum from a potassium bromide disc, the materials being ground together by hand; there was no mention of subsequent heating. The Sadtler Pharmaceutical collection includes a spectrum of a potassium bromide disc in which the material is apparently amorphous and which is almost indistinguishable from the corresponding spectrum of digoxin. A spectrum of a liquid paraffin mull has been published in the Sadtler Standard collection. No evidence of polymorphism was found on recrystallisation.

Digoxin. The spectra of the Authentic Specimen in potassium bromide and in liquid paraffin mull, agree with those published by Bell (1960) and by Hayden & others (1962), though the latter authors stated that the characteristic spectrum was produced only when the disc was heated. The Sadtler Pharmaceutical collection has a spectrum of apparently

amorphous material not readily distinguishable from digitoxin (see above). Digoxin was virtually insoluble in chloroform and acetone, but recrystallisation from hot ethanol gave material with a spectrum identical to that of the original sample except for an additional small peak at 1656 cm^{-1}. This disappeared on heating and was presumably due to hydration.

Dimethisterone. Only one crystalline form was identified, the original material being recovered unchanged from cold acetone. All other solvent treatments gave a glassy solid with a different spectrum. X-ray diffraction confirmed that this was amorphous.

Ethinyloestradiol. Spectra of two crystalline forms have been recorded as mulls by Röpke & Neudert (1959), who also state that, when the potassium bromide disc technique is used, both forms give the same spectrum, which is similar to the solution spectrum. However, the same authors (Neudert & Röpke, 1957) have published a spectrum obtained from a potassium bromide disc which corresponds to their form A. On the other hand, Hayden & others (1962), using a potassium bromide disc, obtained a spectrum intermediate between the two forms, and an identical spectrum has been published by Carol (1957).

This apparent inconsistency in results is explained by the present work in which it was found that form A, the form in which the B.P. Authentic Specimen was received, is stable when ground with potassium bromide, whereas form B, obtained from hot ethanol or cold acetone evaporation, is unstable. A sample of the U.S.P. Reference Standard material was shown by both infrared spectrum and X-ray diffraction pattern to be a mixture of both forms, with B predominating. A third, amorphous form was obtained from hot acetone, and from the similarity in spectrum it seems probable that it is this which is present in pressed discs prepared from form B. Treatment with cold chloroform, recommended in the original leaflet (ASL. 18) issued with the B.P. Authentic Specimen, sometimes gave an amorphous product containing residual chloroform, and the use of solution spectra, as advised in the current leaflet (ASL. 18/2), therefore seems preferable.

Fludrocortisone acetate. Three crystalline forms and one amorphous form have been identified. No simple treatment consistently gave the same form, and interconversion between some of the forms appeared to take place spontaneously. It was found that a reasonably reproducible spectrum could be obtained by evaporating a chloroform solution in a beaker at room temperature to give a glassy material containing chloroform; on heating in an oven at 100° for 15 min the residual solvent was removed, leaving a crystalline product. The Sadtler Pharmaceutical collection includes spectra of two samples: one corresponds to the Authentic Specimen and the other to the amorphous form.

Fluocinolone acetonide. Two crystalline forms were obtained. Treatment with hot ethanol converted the original material into the second form, whilst all other solvent treatments gave mixtures of the two forms. A spectrum was also recorded from a solution in chloroform.

Hydrocortisone. The original form was recovered unchanged from hot ethanol and hot and cold acetone. On evaporation of chloroform solution the spectrum was unchanged except for additional bands at 761 and 752 cm^{-1}, apparently due to residual chloroform. X-ray diffraction measurements however, showed that it was a different crystalline form; on heating it lost chloroform and reverted to the original form. Spectra published by Antonucci, Bernstein, Heller, Lenhard, Littell & Williams (1953), Roberts, Gallagher & Jones (1958), Hayden & others (1962), and the Sadtler Pharmaceutical collection, and band positions quoted by Heller (1959) all correspond to the original form. However, a spectrum published by Hayden (1955), described as 17-hydroxycorticosterone, differs from the forms obtained in the present work.

Methylprednisolone. Two forms have been described by Higuchi, Lau, Higuchi & Shell (1963). The Authentic Specimen corresponded to their form I, which was also recovered from cold acetone and chloroform. Hot acetone yielded form II, and other solvent treatments gave mixtures. The spectrum in the Sadtler Pharmaceutical collection is of form I.

Norethandrolone. The original material was recovered unchanged from hot chloroform. Hot ethanol treatment gave a second form and all other treatments gave mixtures. A spectrum of the second form is included in the Sadtler Pharmaceutical collection.

Norethisterone. We have found no evidence of polymorphism, but the only previously published spectrum, that in the Sadtler Pharmaceutical collection, differs from that of the Authentic Specimen.

Norethynodrel. This substance, which has a double bond in the 5, 10-position, readily undergoes isomerisation to norethisterone where the double bond is between C_4 and C_5. The possibility that such isomerisation might be caused by grinding with halide or liquid paraffin has been examined but no evidence that it occurs to any significant extent has been obtained. Similarly, solution in chloroform B.P. or in methylene chloride does not induce isomerisation, but there is evidence that it begins to occur in chloroform containing as little as 0·005% w/v of hydrochloric acid. In more acid solution isomerisation may take place rapidly and to a marked extent. It is for this reason that the leaflet accompanying the B.P.C. Authentic Specimen directs that solution spectra be recorded using a solution in chloroform containing 0·1% of pyridine.

In the investigation of solid state spectra it was found that the original material was recovered unchanged from hot ethanol, hot chloroform and hot methylene chloride (the latter solvent is that recommended for recrystallisation in the leaflet issued with the B.P.C. Authentic Specimen). Evaporation of acetone, either hot or cold, yielded a second form which differs from the original only in having a strong absorption at 1645 cm^{-1}. This suggests that it may be a hydrate.

Prednisolone. The B.P. Authentic Specimen was received in one form (designated A); a sample of the U.S.P. Reference Standard material was received in a second form (B). Another commercial sample was shown by infrared spectrum and X-ray diffraction pattern to be a mixture of the

two forms. Form B was converted to form A by evaporation of hot acetone or hot chloroform solutions. When form A was dissolved in methanol and heated on a water-bath the resulting material was a mixture; a second treatment with methanol further increased the proportion of form B, though form A was still present. Heating the methanol solution under reflux for 20 min before evaporation still gave a mixture. On the other hand, when form B was dissolved in methanol and the solvent evaporated the product was entirely form B. Two recrystallisations from ethanol converted form A completely into form B.

The spectrum published by Roberts, Gallagher & Jones (1958) is of form A. Hayden & others (1962), using material recrystallised from ethanol, give a spectrum of a mixture in which form A predominates. The Sadtler Pharmaceutical collection contains two spectra, one similar to that of Hayden & others, the other predominantly of form B.

Prednisolone trimethylacetate. Three forms were identified. The original form (A) was recovered from hot acetone treatment of forms A and B (conversion from C was not investigated). Forms B and C were obtained from hot ethanol and hot chloroform respectively, but in each case required two recrystallisations. A solution spectrum was also obtained using a saturated (5%) solution in chloroform in a 0·2 mm cell.

Prednisone. The original form was recovered unchanged from all solvent treatments except cold chloroform. This gave a second crystalline form with a spectrum which differed from the original in several respects, particularly in the presence of a strong band at 749 cm^{-1}, suggesting the presence of chloroform in the crystal. Spectra published by Roberts & others (1958), Hayden & others (1962) and in the Sadtler Pharmaceutical collection all correspond to the first form.

Prednisone acetate. The original form was recovered from hot ethanol. Hot acetone and hot and cold chloroform all yielded a second form, while cold acetone gave a mixture. A solution spectrum was also obtained.

Progesterone. The original form (designated A), which has a strong absorption at 870 cm^{-1}, was recovered from cold chloroform. Hot ethanol and cold acetone gave form B, in which the strong band is at 864 cm^{-1}, while other solvent treatments gave mixtures. Spectra published by Neudert & Röpke (1957), Meda (1958) and the Sadtler Standard and Sadtler Pharmaceutical collections are all of form A; Morcillo & Alduma (1957) give form B; and spectra of mixtures in which form B predominates are given by Hayden & others (1962), who obtained their material by evaporating an ethanol solution, and by Furchgott, Rosenkrantz & Shorr (1947). The latter authors, working before the advent of the pressed disc technique, used a film deposited on a rock salt plate from pyridine solution. Progesterone is readily soluble in chloroform, and spectra can also be obtained from solutions in carbon tetrachloride and carbon disulphide.

Spironolactone. The original form was not recovered from any of the five solvent treatments. A second crystalline form was obtained from cold acetone, but all other solvent treatments gave an amorphous, glassy

material with a different spectrum. A potassium bromide disc of the original material gave a spectrum corresponding to the amorphous form. A spectrum was also obtained in chloroform solution. The Sadtler Pharmaceutical collection contains a spectrum which corresponds to a mixture of the two crystalline forms.

Testosterone. Three forms were encountered, two of which show spectral differences corresponding to those between the two forms of progesterone. The original form (A) has an absorption band at 870 cm^{-1}, and was recovered unchanged from hot acetone and hot chloroform. Cold acetone gave form B, in which the strong band is at 864 cm^{-1}, whilst form C, in which it has shifted to 881 cm^{-1}, was obtained together with form A from cold chloroform. Hot ethanol gave a mixture of forms A and B.

Published spectra show some confusion: the spectrum in the Sadtler Pharmaceutical collection shows a mixture of forms A and C, the Sadtler Standard collection includes two spectra, a rather poor mull of form A (No. 727) and a potassium bromide disc which shows a mixture of form B with a little of form A (No. 13203); a similar mixture is given by Furchgott, Rosenkrantz & Shorr (1946) using a film deposited from pyridine; the spectrum of Morcillo & Alduma (1957) is apparently of form B; that of Neudert & Röpke (1957) is of form A. Rosenkrantz, Potvin & Skogstrom (1958) use a band at 872 cm^{-1} for the quantitative estimation of testosterone in potassium bromide discs. This presumably refers to form A, but errors could obviously arise if other forms were present.

As with progesterone, spectra may be recorded in solution in chloroform, carbon tetrachloride and carbon disulphide.

Triamcinolone. The original form (A) was not recovered unchanged, though cold acetone treatment produced a spectrum fairly close to the original. Hot ethanol and hot acetone both gave a second form (B). Evaporation of a methanol solution is recommended in the leaflet issued with the B.P.C. Authentic Specimen, but this seems a bad choice as it gave mixtures of varying proportions of Forms A and B, starting from either A or B. A spectrum published in the Sadtler Pharmaceutical collection is mainly form A, but contains some impurity.

General discussion

The results quoted above show that sample preparation techniques can have a profound effect on the spectra of steroids. The inconsistency of published spectra confirms this, and serves to emphasise the necessity for comparison between the sample and an Authentic Specimen under identical conditions as the basis of any identification by infrared spectroscopy.

Experienced workers in this field (Jones & Dobriner, 1949; Page, 1957) have recommended that whenever possible infrared spectra of steroids should be recorded in solution, and with compounds which exhibit polymorphism this is undoubtedly advisable. Nevertheless, if two samples are found to give identical spectra in the solid phase, this constitutes reasonable proof of identity and no recourse to solutions is required.

Difficulty arises only when samples, which are thought to be of the same substance, give different spectra in the solid phase. Spectra recorded in solution will soon resolve any doubts, but unfortunately many of the physiologically important steroids are only sparingly soluble in the commonly used solvents, and the use of solid phase spectra is often unavoidable.

The normal way to overcome the effects of polymorphism is to convert both samples into the same form by recrystallisation from the same solvent. However, where the presence of impurities may be in question it is necessary to recover the whole of the material and solutions must therefore be evaporated to dryness. With some solvents this may lead to the production of two different crystalline forms from the same solution, and such solvents should be avoided. In many instances the temperature at which the solvent is removed can also affect the form of the resulting solid. To ensure reproducibility it has been found necessary to prescribe individual solvent treatments for the insoluble substances which exhibit polymorphism, as follows: Betamethasone: Dissolve in chloroform and evaporate on a water-bath. Fludrocortisone acetate: Dissolve in chloroform in a beaker and evaporate in a current of air at room temperature; heat residue in oven at 100° for 15 min. Hydrocortisone: Dissolve in acetone and evaporate on a water-bath. Prednisolone: Dissolve in acetone or chloroform and evaporate on a water-bath. Prednisone: Dissolve in acetone and evaporate on a water-bath. Triamcinolone: Dissolve in acetone and evaporate on a water-bath.

With regard to sample form, mulls in liquid paraffin gave more consistent spectra than potassium halide discs, particularly with the substances marked † in Table 1, and this technique appears preferable for comparison purposes (bearing in mind the presence of absorptions due to the liquid paraffin). On the other hand, prednisolone sodium phosphate was difficult to grind in liquid paraffin and gave a much better spectrum in a potassium bromide disc, and the same was true to a lesser extent with hydrocortisone sodium succinate.

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The response to transmural stimulation of isolated arterial strips and its modification by drugs

GAVIN PATERSON

Transmural electrical stimulation of isolated aortic strips of rabbits, induced contractions of the muscle which were blocked by concentrations of cocaine and lignocaine which augmented the response to noradrenaline. The adrenergic neurone blocking agents bretylium, guanethidine and bethanidine also blocked selectively the response to transmural stimulation. With bretylium and guanethidine the responses to noradrenaline were augmented. α-Receptor blockade with piperoxan, phentolamine, phenoxybenzamine and dihydrogenated ergot alkaloids also blocked the responses to transmural stimulation. Cocaine in low concentration and dexamphetamine augmented the responses to electrical stimulation. They also delayed onset of block with adrenergic neurone blocking drugs and reversed this when it was already established. Hexamethonium and pentolinium, in concentrations which in other tissues are known to cause complete blockade of ganglionic transmission, had no effect on electrical stimulation. It is concluded that the responses to electrical stimulation are the result of excitation of post-ganglionic adrenergic axons in the walls of the arterial preparation. Strips of common carotid, superior mesenteric, renal, pulmonary and common iliac arteries of the rabbit gave responses to electrical stimulation similar to those of the aorta.

STRIPS of arteries have for many years been used for the study of the physiological and pharmacological responses of vascular smooth muscle (see Furchgott, 1955; Bohr, 1964; Green & Boura, 1964). A nerve-muscle preparation has been described for the pulmonary artery of the rabbit (Bevan, 1962; Bevan & Su, 1964) but, apart from this, attempts to obtain an uncomplicated neurogenic response in isolated arterial preparations have been unsuccessful (Furchgott, 1952; Leonard, 1957) mainly because of the form and parameters of electrical stimulation which have been necessary for eliciting a contraction in these preparations. Where square wave impulses were used it was necessary to use pulse durations of 10 to 50 msec and 50 to 100 V to evoke contractions, but it was found that responses to other forms of stimulus were subsequently depressed (Leonard, 1957). This led to the use of low voltage alternating current (50 to 60 cycles/sec) for inducing reproducible responses from arterial strips (Furchgott, 1952; Leonard, 1957; Gillis & Yates, 1963; Ghosh & Roddie, 1964). The contraction induced in this way had two components: during the period of stimulation a rapid contraction occurred; on cessation of stimulation a second, slower contraction appeared from which the muscle recovered slowly. The first phase appeared to be the result of direct stimulation of the muscle, but the second phase was ascribed by Furchgott to the release of an adrenaline-like substance in the arterial wall, since dibenamine blocked this phase of the response. This concept was strengthened by the results of Gillis & Yates (1963) using piperoxan, bretylium or reserpine, all of which abolished the second phase.

The introduction of transmural stimulation of smooth muscle (Paton, 1955) extended the possibilities for neurogenic electrical stimulation of

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isolated tissues and it is with this form of stimulation that the present experiments are concerned.

When artery strips are excited by transmural stimuli of short pulse duration, reproducible responses can be obtained which on pharmacological analysis are free from a "direct" component of stimulation and are susceptible to modification by substances affecting post-ganglionic adrenergic nerve mechanisms.

Experimental

Rabbits, 1·5 to 3 kg, were killed by a blow to the back of the neck. The thorax and abdomen were opened and the entire descending aorta dissected into cold Krebs solution gassed with 95% oxygen and 5% carbon dioxide. The aorta was extended under tension between two small artery clips secured by lengths of cotton. This facilitated clearance of fatty and connective tissue and the subsequent dissection. An incision was made about 2 mm from the cardiac end of the vessel and a spiral strip cut measuring about 3 mm wide by about 5 cm long. The intact ring of the artery at the cardiac end was used to fix the preparation on a perspex rod mount. The other end of the strip was attached to an isotonic frontal lever, of load 4 g and magnification $\times 12$, writing on a smoked paper. The stimulating electrodes were also an integral part of the assembly on which the muscle strip was mounted (for details see Birmingham & Wilson, 1963; Paterson, 1965) and consisted of two parallel wires cemented vertically on two sides of a Perspex channel with the aortic strip suspended between the wires. The muscle was arranged so that it presented its flat surface to the electrodes and was immersed at 37° in 75 ml Krebs solution gassed with 95% oxygen and 5% carbon dioxide.

ELECTRICAL STIMULATION

Some preliminary experiments were necessary to establish the optimum parameters of stimulation. The findings of these are described more fully in Results but optimum conditions for electrical stimulation were found to be with square wave impulses of 300 μsec duration and 100 to 120 V at a frequency of 20 or 25/sec. Both Palmer H44 and Multitone stimulators gave satisfactory responses. The preparation was stimulated for 1 min every 40 min, timed by a cam-operated micro-switch device. Responses to drugs were usually interposed between periods of transmural stimulation and were timed to occur 20 min after the end of stimulation. After setting up the preparation the responses to drugs and transmural stimulation increased for up to 2 hr during which time the muscle slowly relaxed. Responses were then uniform for a further 6 to 8 hr. When strips from arteries other than the aorta were used they were prepared in the same way.

Drugs used were: piperoxan hydrochloride; phentolamine hydrochloride; phenoxybenzamine hydrochloride; lignocaine hydrochloride; cocaine hydrochloride; dihydroergotamine methanesulphonate; bretylium

tosylate; guanethidine hemisulphate; bethanidine sulphate; guanoxan sulphate; hexamethonium bromide; pentolinium tartrate; dexamphetamine sulphate; (−)-noradrenaline bitartrate; angiotensin. Concentrations are expressed as final bath concentration (g/ml) in terms of the base.

Results

ELECTRICAL STIMULATION. EFFECTS OF VARYING THE FREQUENCY ON THE RESPONSE OF THE ARTERIAL STRIP TO TRANSMURAL STIMULATION

With a pulse of 300 μsec duration and 100 V, aortic strips were stimulated for 30 sec at frequencies of 1, 2, 5, 10, 20 and 50/sec, with 20 min between trains of stimuli. The contraction with a frequency of 1/sec was less than 10% of that at 50/sec in one preparation and in two out of three preparations was less than 1%. It was found that the lowest frequency at which a consistently useful contraction was obtained was 20/sec. The contraction at a frequency of 50/sec was always larger than that at 20/sec, but the height of contraction at 50/sec declined in 2–3 hr and was often accompanied by changes in "tone" of the preparation, whereas at 20/sec, responses were uniform for upwards of 6 hr.

EFFECTS OF VARYING THE INTERVAL BETWEEN STIMULI

Aortic strips were stimulated for 15 sec every 10 min with pulses of 300 μsec and 100 V at a frequency of 20/sec. Reproducible contractions were obtained, but relaxation to resting tension was incomplete and a raised base-line response was produced. When stimulated for 30 sec every 20 min using the same stimulus parameters, the strip gave larger contractions, but relaxation was almost always complete between periods of stimulation. This was the form of stimulation chosen where responses to stimulant drugs such as noradrenaline were not recorded. In most experiments, however, responses to noradrenaline were interpolated between responses to transmural stimulation and in these circumstances the period of electrical excitation was 1 min in every 40 min. The concentration of the noradrenaline was adjusted to give a contraction similar in height to that of transmural stimulation.

EFFECTS OF LOCAL ANAESTHETIC DRUGS

The two drugs chosen were lignocaine and cocaine. These had been shown to have only about one-fifth of the muscle-depressant properties of procaine on the rabbit isolated aorta (Åström, 1964). Lignocaine caused block of responses to transmural stimulation ranging from 30% block with 5×10^{-6} to 100% block with 4×10^{-5}. With these concentrations the response to noradrenaline was potentiated (with 4×10^{-5} lignocaine, the contraction with 10^{-8} noradrenaline was nearly doubled). With 2×10^{-5} and 4×10^{-5} cocaine, 75% and 90% block respectively of transmural stimulation were obtained, while again the response to noradrenaline was enhanced. Cocaine 5×10^{-6} enhanced the responses both to transmural stimulation or added noradrenaline. Both enhancement

and blockade by the local anaesthetics were quickly reversed on washing the preparation.

EFFECTS OF SUBSTANCES BLOCKING α-RECEPTORS

Piperoxan (5×10^{-7} to 2×10^{-6}) caused partial to complete block of the responses to transmural stimulation and to noradrenaline. The effect of 10^{-6} piperoxan on transmural stimulation is illustrated in Fig. 1. The block reversed on washing.

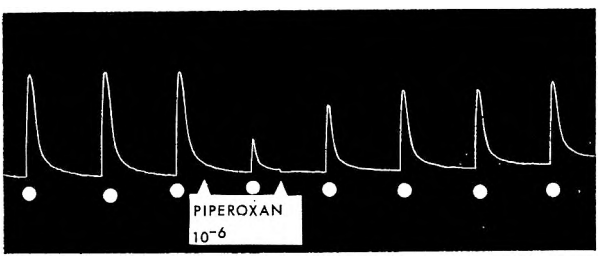

FIG. 1. Rabbit aortic strip. The blockade of responses to transmural stimulation by piperoxan. At the white spots the preparation was stimulated transmurally at a frequency of 20/sec for 30 sec every 20 min. The duration of the square wave pulse was 300 μsec and voltage was 120 V. Piperoxan (10^{-6} final bath concentration) was added to the bath and remained in contact with the muscle for the period indicated by the arrows. It was then washed out.

Phentolamine (10^{-7} to 10^{-6}) again blocked transmural stimulation and added noradrenaline, but, on washing, recovery was slower than with piperoxan (Fig. 2). Phentolamine (10^{-7}) did not alter the responses of the preparation to 2×10^{-9} angiotensin at a time when 2×10^{-9} noradrenaline was completely blocked and transmural stimulation was reduced to 10% of normal. Phenoxybenzamine (5×10^{-7} to 10^{-6}) blocked irreversibly the responses to both transmural stimulation and to

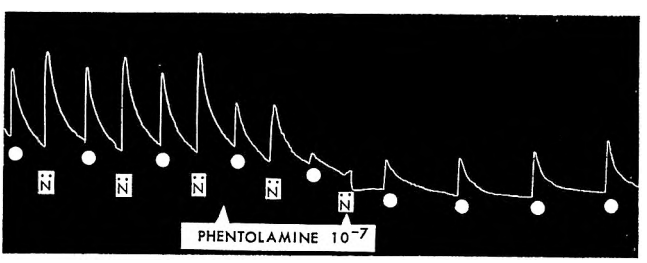

FIG. 2. Rabbit aortic strip. The blockade of responses to transmural stimulation and to noradrenaline by phentolamine. At the white spots the preparation was stimulated transmurally at a frequency of 20/sec for 1 min every 40 min. The duration of the square wave pulse was 300 μsec and voltage was 100 V. At N, noradrenaline (10^{-8}) was added to the bath and washed out after 4 min contact; the duration of contact with the muscle is indicated by the two dots. Phentolamine (10^{-7}) was added to the bath and remained in contact with the muscle for the period indicated by the arrows. It was then washed out. Concentrations are expressed as final bath concentration.

noradrenaline. Onset of block occurred in less than 10 min and despite several changes of Krebs over a 4 hr period, no recovery of adrenergic function was detectable. Again responses to angiotensin could still be elicited when both transmural stimulation and noradrenaline were completely blocked with 10^{-6} phenoxybenzamine.

With dihydroergotamine (DHE; 10^{-6}, 2×10^{-6}) and Hydergine (2×10^{-6}) block was complicated in four out of six experiments by a slowly developing contraction, but 70% block was obtained with 2×10^{-6} DHE and 50% with 2×10^{-6} Hydergine.

ADRENERGIC NEURONE BLOCKING AGENTS

Bretylium (5×10^{-7} to 2×10^{-6}) or guanethidine (2×10^{-7} to 10^{-6}) caused progressive blockade of transmural stimulation accompanied by a potentiation of the response to noradrenaline (Figs 3 and 4). On

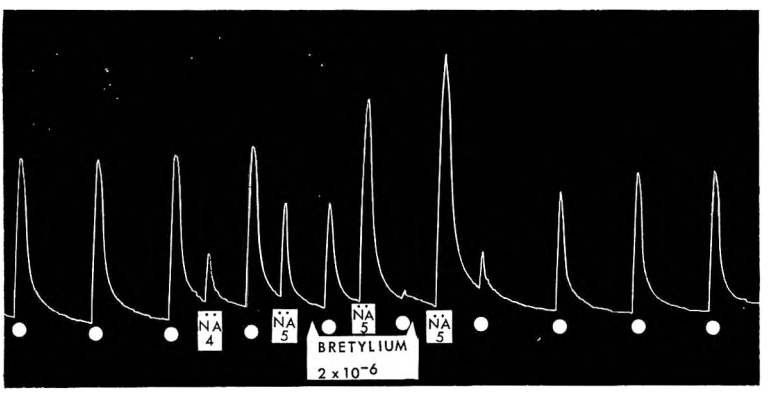

FIG. 3. Rabbit aortic strip. The blockade of responses to transmural stimulation, and enhancement of response to noradrenaline, by bretylium. At the white spots the preparation was stimulated transmurally at a frequency of 20/sec for 1 min every 40 min. The pulse width was 300 μsec and the voltage was 100 V. At NA4 and NA5, noradrenaline was added to the bath to give final concentrations of 4×10^{-9} and 5×10^{-9} respectively. The duration of contact of the noradrenaline with the muscle was 4 min and is indicated by the dots. Bretylium (2×10^{-6} final bath concentration) was added to the bath and remained in contact with the muscle for the period indicated by the arrows. It was then washed out.

changing the bathing fluid the block caused by bretylium was reversed in 1 hr, but that with guanethidine was unchanged or only slightly reversed after washing for 4 hr. With higher concentrations of bretylium or guanethidine (5×10^{-6}) complete block of transmural stimulation was accompanied by a slowly developing contraction which reversed on washing. Bethanidine (10^{-7} to 10^{-6}) caused block of transmural stimulation, but with the concentrations used no increase in the action of noradrenaline (Fig. 5).

Guanoxan (5×10^{-7} to 2×10^{-6}) caused a decrease in the effects of both transmural stimulation and noradrenaline. On washing, the response to noradrenaline recovered quickly, but transmural stimulation was still reduced $1\frac{1}{2}$ hr after washing (Fig. 6).

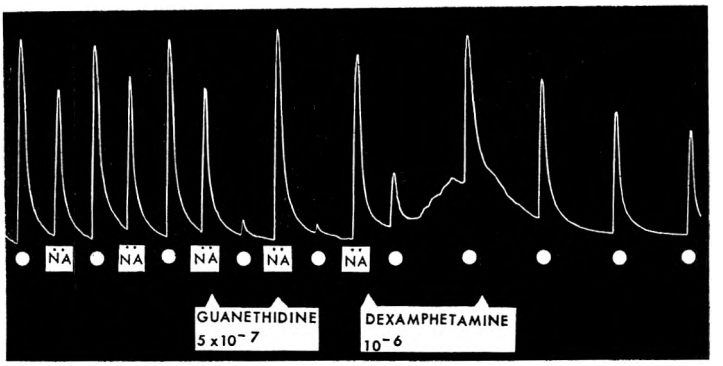

FIG. 4. Rabbit aortic strip. The blockade of responses to transmural stimulation, and enhancement of response to noradrenaline, by guanethidine. At the white spots the preparation was stimulated at a frequency of 20/sec for 1 min every 40 min. The pulse width was 300 μsec and the voltage was 100 V. At NA, noradrenaline (5×10^{-9}) was added to the bath and washed out after 4 min contact; the duration of contact with the muscle is indicated by the dots. Guanethidine (5×10^{-7}) was added to the bath and remained in contact with the muscle for the period indicated by the arrows. It was then washed out. Subsequently dexamphetamine (10^{-6}) was added to the bath for the period indicated by the arrows and was then washed out. Concentrations are expressed as final bath concentration.

REDUCTION OF ADRENERGIC NEURONE BLOCK BY DEXAMPHETAMINE AND BY COCAINE

Dexamphetamine (2×10^{-7} to 5×10^{-6}) reversed the sustained block of responses to transmural stimulation seen after washing out guanethidine (Fig. 4), bethanidine or guanoxan (Fig. 6). The reversal was usually accompanied by a slowly developing contraction of the aortic strip (Figs 4 and 6). On washing out, the reversal was only partly sustained (Fig. 4),

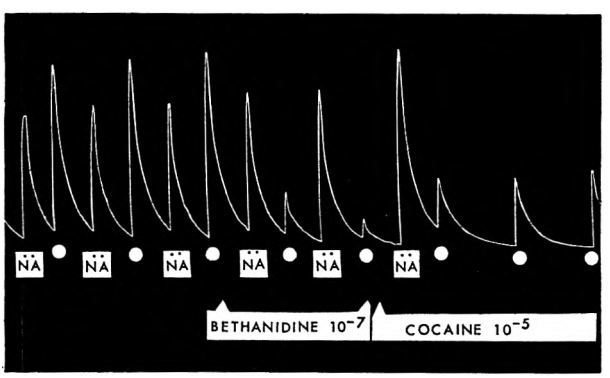

FIG. 5. Rabbit aortic strip. The blockade of responses to transmural stimulation by bethanidine. At the white spots the preparation was stimulated at a frequency of 20/sec for 1 min every 40 min. The pulse width was 300 μsec and the voltage was 100 V. At NA, noradrenaline (3×10^{-9}) was added to the bath and washed out after 4 min contact; the duration of contact with the muscle is indicated by the two dots. Bethanidine (10^{-7}) was added to the bath and remained in contact with the muscle for the period indicated by the arrows. It was then washed out. Cocaine was subsequently added to the bath at the arrow and left in the bath until the end of the experiment. Concentrations are expressed as final bath concentration.

but even when the dexamphetamine remained in the bath there was some reduction in the height of contraction (Fig. 6).

Three combinations of guanethidine and dexamphetamine (2×10^{-7} guanethidine and 2×10^{-7} dexamphetamine; 2×10^{-7} guanethidine and 4×10^{-7} dexamphetamine; 4×10^{-7} guanethidine and 4×10^{-7} dexamphetamine) were compared with the effect of 2×10^{-7} guanethidine alone, on responses to transmural stimulation (30 sec every 20 min) in four aortic strips from the same rabbit. Each combination was left in contact with one strip and then washed out. In the strip subjected to guanethidine only, full action developed in 30 min and was not reversed on washing. In all three preparations which had dexamphetamine added, responses to transmural stimulation were unchanged or slightly enhanced until the drugs were washed out; thereafter block of the responses developed in all three strips, but in none was it as complete as in the strip which had guanethidine only.

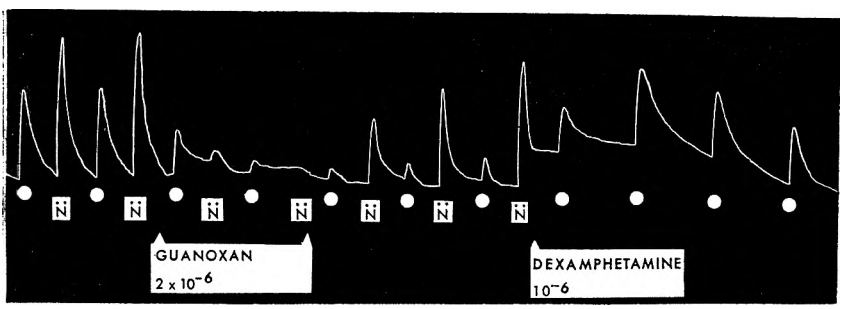

FIG. 6. Rabbit aortic strip. The blockade of responses to transmural stimulation and noradrenaline by guanoxan. At the white spots the preparation was stimulated transmurally at a frequency of 20/sec for 1 min every 40 min. The stimulus was 200 μsec and 100 V. At N, noradrenaline (5×10^{-9}) was added to the bath and washed out after 4 min contact; duration of contact is indicated by the two black dots. Guanoxan (2×10^{-6}) was added to the bath and remained in contact with the muscle for the period indicated by the arrows. Dexamphetamine (10^{-6}) was added to the bath at the arrow and remained in the bath for the remainder of the experiment. Concentrations are expressed as final bath concentrations.

Cocaine (5×10^{-6} to 10^{-5}) similarly reversed the persistent block caused by guanethidine (4×10^{-7}) and when given with the guanethidine prevented the onset of block until the drugs were washed out. Cocaine (5×10^{-6}) or dexamphetamine ($2{\cdot}5 \times 10^{-7}$) when given alone, increased the responses to transmural stimulation.

GANGLION BLOCKING AGENTS

Concentrations of hexamethonium and pentolinium up to 5×10^{-5} had no effect on transmural stimulation of aortic strips.

STRIPS FROM ARTERIES OTHER THAN THE AORTA

Arterial strips have been prepared from segments of the pulmonary, common carotid, renal, superior mesenteric and common iliac arteries of the rabbit and have yielded suitable responses on transmural stimulation.

These responses have not been fully investigated, but have not differed from those of the aorta to adrenergic neurone blockade. Strips from pulmonary arteries relaxed much more quickly after stimulation than did aortic strips.

ARTERIAL STRIPS FROM OTHER SPECIES

Aortic strips from guinea-pigs and carotid artery strips from cats also gave responses to transmural stimulation, but these have not been fully investigated.

Discussion

Isolated arterial muscle strips were stimulated transmurally with pulses of short duration if the strips were mounted between parallel wire electrodes. The mechanism by which responses to transmural stimulation were induced may be inferred from a consideration of the following.

The pulse width which gave optimal response of the tissue, 200 to 300 μsec, has been generally found, although not exclusively so, to stimulate nervous structures only. The local anaesthetic drugs, lignocaine and cocaine, in concentrations which did not block the action of noradrenaline on these preparations, abolished transmural stimulation. Substances which blocked the actions of noradrenaline on α-receptors in these strips also blocked concomitantly the responses to transmural stimulation; this was found with phentolamine, piperoxan, phenoxybenzamine, dihydroergotamine or Hydergine. The adrenergic neurone blocking agents, bretylium, guanethidine and bethanidine had a selective blocking effect on transmural stimulation. Also, cocaine or dexamphetamine potentiated the responses both to noradrenaline and transmural stimulation, and finally, the ganglion blocking agents hexamethonium and pentolinium had no effect on transmural stimulation.

On these counts the mechanism of the transmural stimulation was considered to be neurogenic and probably sited at post-ganglionic adrenergic nerve elements. One of the strongest pieces of evidence for this was the selective blockade with bretylium, guanethidine or bethanidine. These drugs have been shown to have selective blocking effects at adrenergic nerve endings only (Boura & Green, 1959, 1963; Maxwell, Plummer, Schneider, Povalski & Daniel, 1960).

The identity of the transmitter involved cannot be ascertained from the present experiments, but the catecholamine present in the walls of blood vessels is almost entirely noradrenaline and is associated with neural networks (Schmiterlöw, 1948; Falck, 1962; Norberg & Hamberger, 1964), so that it seems likely that this amine will be primarily concerned in transmural stimulation.

Potentiation of the actions of noradrenaline were seen with lignocaine, cocaine, bretylium or guanethidine. It seemed likely that these substances inhibited the uptake of noradrenaline into catecholamine stores. Muscholl (1961) has shown this to be so for cocaine, and Iversen (1965) confirmed this action and extended the observation to a number of other substances including bretylium and guanethidine. Bretylium and guanethidine also had a contractile action on aortic strips, but this was seen here to occur

with concentrations several times greater than those which caused block of transmural stimulation and potentiation of the action of noradrenaline. Kirpekar & Furchgott (1964) found that this contractile action of bretylium was due to release of noradrenaline from stores in the tissue since reserpinised strips did not respond to bretylium unless previously incubated with noradrenaline. Maxwell, Daniel, Sheppard & Zimmerman (1962) came to a similar conclusion for the action of guanethidine. Guanethidine is also known to deplete tissue stores of noradrenaline, an action which was found to be unrelated to adrenergic neurone block (Cass & Spriggs, 1961).

Reversal by dexamphetamine of the block of transmural stimulation caused by guanethidine, guanoxan and bethanidine was particularly striking where the block persisted after washing out these drugs. Although dexamphetamine has been shown here to have a potentiating action of its own, this would not in itself be sufficient to explain the reversal seen here, or the reduction in the action of guanethidine when the two drugs were administered together. These findings support the hypothesis that to some extent competition for a common site between dexamphetamine and guanethidine may explain this action (Day, 1962; Day & Rand, 1963).

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Application of the Ferguson principle to the antimicrobial activity of quaternary ammonium salts

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The activity of three quaternary ammonium salts, dodecyltrimethylammonium chloride, dodecyldimethylethylammonium chloride and dodecylpyridinium chloride, against *M. aureus*, *E. coli* and *C. albicans* has been determined and correlated with the surface properties of these compounds. The Ferguson principle has been applied by using surface concentration to estimate thermodynamic activity. Results obtained for the three quaternary ammonium salts and a particular micro-organism species were found to be in good agreement with each other when microbiological and surface studies were conducted under identical conditions.

SINCE the discovery of the antimicrobial activity of alkylbenzyldimethylammonium chlorides by Domagk (1935), quaternary ammonium salts have received wide recognition as effective germicides. They are effective against Gram-positive and Gram-negative bacteria, as well as a wide range of fungi (Kull, Eisman, Sylwestrowicz & Mayer, 1961). The antimicrobial activity of these compounds has been related to their surface activity, as demonstrated by surface tension lowering (Zissman, 1957), and critical micelle concentrations (Cella, Eggenberger, Noel, Harriman & Harwood, 1952). They have also been reported to exert their effect by altering the microbial cytoplasmic membrane (Hotchkiss, 1946, Gale & Taylor, 1947, Gilby & Few, 1957).

We set out to determine whether the Ferguson principle (Ferguson, 1939) is applicable to these systems when the surface properties of the compounds under consideration are used to obtain thermodynamic activities. To quantitatively correlate surface tension data with antimicrobial activities, these should be assessed in the same conditions as those in which the surface properties are measured otherwise a number of changes in the environment of the antimicrobial agent and of the micro-organism would have to be taken into account. For example, the ionic strength of the two media may be different and affect the surface tension as well as the growth of the micro-organism; changes in temperature, besides having an effect on the micro-organisms, may alter the surface properties of the compound under investigation; an ingredient in a nutrient medium, e.g., protein, may complex with the compound under investigation, thereby either antagonising or potentiating the antimicrobial activity (Few, Ottewell & Parreira, 1955), or altering the surface properties of the compound (Smith, Shay & Doorenbos, 1964). To avoid introducing unnecessary variables, microbial experiments were made in 0·1M potassium chloride solutions at 25°, the same conditions used to measure the surface properties of the compounds under investigation.

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Experimental

Quaternary ammonium salts. Three compounds, each having the same chain length and counterion, but differing in their polar group, were chosen. These were: dodecyltrimethylammonium chloride (DTAC); dodecyldimethylethylammonium chloride (DEAC); and dodecylpyridinium chloride (DPC). The methods of preparation and purification have been reported previously (Weiner & Zografi, 1965).

Surface tension measurements. The surface tensions and critical micelle concentrations of these compounds in 0·1M potassium chloride at 25° were measured by the drop-volume method. Surface concentrations, expressed as molecules/cm^2, were calculated from the Gibbs adsorption equation. The details of these experiments have been reported previously (Weiner & Zografi, 1965).

Choice of micro-organisms. Three species of micro-organisms, purchased from American Type Culture Collection, were used in this investigation. These were: *Micrococcus pyogenes* var. *aureus* 209 (ATCC No. 6538); *Escherichia coli* (ATCC No. 9661), and *Candida albicans* (ATCC No. 752). The cultures were examined microscopically at the conclusion of the investigation, and no apparent contaminants were observed.

MICROBIOLOGICAL TECHNIQUES

Micrococcus pyogenes var. *aureus* 209. A test tube containing 10 ml of nutrient broth (Difco) was inoculated with a 4 mm loopful of culture. This tube was incubated at 37° for 24 hr. Five ml of this mixture was then transferred to a 500 ml Erlenmeyer flask containing 250 ml of nutrient broth. The flask was then incubated in a Gyrotory Shaker at 37°. The incubation was continued until the absorbance of the mixture at 600 mμ was 0·400. This absorbance was found to correspond to a concentration of $3{\cdot}2 \times 10^7$ bacteria/ml. The concentration was determined by the use of standard serial dilution and plating techniques (Burrows, 1959).

Twenty ml of the resulting culture ($3{\cdot}2 \times 10^7$ bacteria/ml) was centrifuged for 5 min, and the supernatant liquid discarded. The discarded liquid was replaced by 20 ml of 0·1M potassium chloride, which was then intimately mixed with the bacteria. The mixture was centrifuged again for 5 min and the supernatant liquid discarded. Enough 0·1M potassium chloride was then added to adjust the concentration to $1{\cdot}0 \times 10^7$ bacteria/ml. 0·5 ml of this mixture was then added to each of a series of tubes containing 4·5 ml of 0·1M potassium chloride; one was control, the others contained various concentrations of the three quaternary ammonium compounds. The contents of each tube were mixed and the tubes were placed in a constant temperature water-bath at 25°. After 15 min, 0·1 ml of each mixture was added to a tube containing 9·9 ml neutralising medium (lecithin, 0·5; polysorbate 80, 3·0 g; nutrient broth, 100 ml). This medium neutralises the antimicrobial activity of quaternary ammonium compounds without significantly affecting the growth of the

organism (Kohn, Gerschenfeld & Barr, 1963). A tube containing about 10 ml of melted nutrient agar (Difco) at 40° was then inoculated with 1 ml of each neutralised mixture. The contents of each tube were mixed and poured into petri dishes. The dishes were incubated at 37° for 24 hr and the number of colonies in each plate was counted. All experiments were made in duplicate.

Escherichia coli. The same technique was used for *E. coli* as for *M. aureus* with the following exceptions. One ml, instead of 5 ml, of the mixture was transferred to a 500 ml Erlenmeyer flask containing 250 ml of nutrient broth. An absorbance of 0·400 at a wavelength of 600 mμ corresponded to a concentration of $9{\cdot}2 \times 10^7$ bacteria/ml. However, the final concentration was also adjusted to $1{\cdot}0 \times 10^7$ bacteria/ml.

Candida albicans. The same technique was used for *C. albicans* as for *M. aureus* with the following exceptions. Sabouraud agar and broth (Difco) were used, the incubation temperature was 25°, and 10 ml of the mixture was transferred to a 500 ml Erlenmeyer flask containing 250 ml of Sabouraud broth. An absorbance at 600 mμ of 0·400 corresponded to a concentration of $1{\cdot}1 \times 10^7$ organisms/ml; the final concentration was adjusted to $1{\cdot}0 \times 10^7$ organisms/ml.

All experiments were made during the logarithmic growth phase of the micro-organism, as previously determined by standard procedures (Burrows, 1959).

Since the antimicrobial activities of the quaternary ammonium compounds were tested in 0·1M potassium chloride solution, it was necessary to determine the effects of this salt solution on the growth of the micro-organisms. Broth-free micro-organisms were inoculated into 0·1M potassium chloride solution and the concentration of the micro-organisms determined at 15 min intervals by standard serial dilution and plating techniques (Burrows, 1959). Over a 30 min interval, potassium chloride did not kill any of the micro-organisms.

Results and discussion

The number of colonies per plate for each concentration of quaternary ammonium compound tested is shown in Table 1. The lowest concentration of each compound that resulted in less than 10 colonies per plate was termed the "minimum effective concentration" (MEC) for that particular organism. This corresponds to a concentration that kills at least 99·9% of the micro-organisms.

The surface activities of the three quaternary ammonium compounds tested are in the same order as the activities against the micro-organisms tested, i.e., DPC>DEAC>DTAC. The interfacial tensions, as well as the corresponding surface concentrations of the quaternary ammonium compounds tested, are compared with their minimum effective concentration values in Table 2. A comparison of these values for the quaternary ammonium compounds for each species of organism, with surface tension, supports Zissmann's findings (1957) that solutions having equal antimicrobial activity have surface tension values of the same order of magnitude. Similarly, solutions having equal antimicrobial activity against a

TABLE 1. ANTIMICROBIAL ACTIVITY OF QUATERNARY AMMONIUM SALTS

Micro-organism	Concentration (molarity) of quaternary ammonium compound	Number of colonies/plate					
		DPC		DTAC		DEAC	
M. aureus	$1{\cdot}0 \times 10^{-4}$	+	+	+	+	+	+
	$1{\cdot}5 \times 10^{-4}$	7	9	+	+	+	+
	$2{\cdot}5 \times 10^{-4}$	0	2	+	+	+	+
	$3{\cdot}0 \times 10^{-4}$	0	0	+	+	0	0
	$4{\cdot}0 \times 10^{-4}$	0	0	+	+	0	0
	$5{\cdot}0 \times 10^{-4}$	0	0	10	5	0	0
	Control	+	+	+	+	+	+
E. coli	$7{\cdot}5 \times 10^{-6}$	+	+	+	+	+	+
	$1{\cdot}0 \times 10^{-4}$	12	7	+	+	+	+
	$1{\cdot}5 \times 10^{-4}$	0	1	+	+	+	+
	$2{\cdot}5 \times 10^{-4}$	0	0	+	+	10	10
	$3{\cdot}0 \times 10^{-4}$	0	0	+	+	0	0
	$4{\cdot}0 \times 10^{-4}$	0	0	0	1	0	0
	Control	+	+	+	+	+	+
C. albicans	$4{\cdot}0 \times 10^{-4}$	+	+	+	+	+	+
	$5{\cdot}0 \times 10^{-4}$	0	1	+	+	+	+
	$6{\cdot}5 \times 10^{-4}$	0	0	+	+	+	+
	$7{\cdot}5 \times 10^{-4}$	0	0	+	+	+	+
	$1{\cdot}0 \times 10^{-3}$	0	0	+	+	+	+
	$1{\cdot}5 \times 10^{-3}$	0	0	+	+	0	0
	$2{\cdot}5 \times 10^{-3}$	0	0	10	3	0	0
	Control	+	+	+	+	+	+

+ Represents 100 colonies or more per plate.

TABLE 2. COMPARISON OF INTERFACIAL PROPERTIES AND THE MINIMUM EFFECTIVE CONCENTRATIONS OF THE QUATERNARY AMMONIUM COMPOUNDS

Micro-organism	Quaternary ammonium compounds	MEC (molarity)	Surface tension (dynes/cm)— Air— 0·1M KCl	Surface concentration $\times 10^2$ (molecules/A^2)— Air— 0·1M KCl
M. aureus	DPC	$1{\cdot}5 \times 10^{-4}$	11·5	1·46
	DTAC	$5{\cdot}0 \times 10^{-4}$	11·4	1·36
	DEAC	$3{\cdot}0 \times 10^{-4}$	9·2	1·25
E. coli	DPC	$1{\cdot}0 \times 10^{-4}$	9·0	1·31
	DTAC	$4{\cdot}0 \times 10^{-4}$	10·2	1·28
	DEAC	$2{\cdot}5 \times 10^{-4}$	8·4	1·19
C. albicans	DPC	$5{\cdot}0 \times 10^{-4}$	19·7	1·83
	DTAC	$2{\cdot}5 \times 10^{-3}$	21·8	1·79
	DEAC	$1{\cdot}5 \times 10^{-3}$	19·1	1·68

particular species of micro-organism have surface concentration values of the same order of magnitude. For example, although there is a four-fold difference in the minimum effective concentration values for DPC and DTAC against *E. coli*, there is less than a 25% difference in any of the corresponding interfacial tension values or surface concentration values. Thermodynamic activities based on critical micelle concentrations and surface concentrations are shown in Table 3. The approach using critical micelle concentrations was previously reported by Ecanow & Siegel (1963), using the data of Cella & others (1952). They considered the thermodynamic activity of a solution that kills 99·99% of the micro-organisms equal to c/CMC, where c is the concentration of the solution, and CMC is the critical micelle concentration. Unfortunately, the microbiological data were not standardised since the time required to kill 99·99% of the micro-organisms tested ranged from 0·35 to 25 min.

TABLE 3. THERMODYNAMIC ACTIVITY NECESSARY FOR 99·9% KILLING OF MICRO-ORGANISMS BY QUATERNARY AMMONIUM COMPOUNDS

Micro-organism	Quaternary ammonium compound	CMC (molarity)	MEC/CMC	$\Gamma_{CMC} \times 10^2$ (molecules/A^2)	$\Gamma_{MEC}/\Gamma_{CMC}$
M. aureus	DPC	$2{\cdot}3 \times 10^{-3}$	0·065	2·02	0·72
	DTAC	$7{\cdot}5 \times 10^{-3}$	0·067	1·94	0·70
	DEAC	$7{\cdot}0 \times 10^{-3}$	0·043	1·81	0·69
E. coli	DPC	$2{\cdot}3 \times 10^{-3}$	0·043	2·02	0·65
	DTAC	$7{\cdot}5 \times 10^{-3}$	0·053	1·94	0·66
	DEAC	$7{\cdot}0 \times 10^{-3}$	0·036	1·81	0·66
C. albicans	DPC	$2{\cdot}3 \times 10^{-3}$	0·22	2·02	0·91
	DTAC	$7{\cdot}5 \times 10^{-3}$	0·33	1·94	0·92
	DEAC	$7{\cdot}0 \times 10^{-3}$	0·21	1·81	0·93

The thermodynamic activities necessary for 99·9% killing of the micro-organisms, based on surface concentrations, are expressed as $\Gamma_{MEC}/\Gamma_{CMC}$, where Γ_{MEC} is the surface concentration at the air-0·1M potassium chloride interface at a bulk concentration corresponding to the minimum effective concentration, and Γ_{CMC} is the surface concentration at the CMC The two thermodynamic activities are not necessarily the same since the standard states employed are different. Whereas Γ_{CMC} represents a saturated "surface solution", the CMC may represent an unsaturated bulk solution.

From the results in Table 3 it is apparent that, whereas thermodynamic activities for a particular species of micro-organism based on bulk concentration are not always in good agreement, activities based on surface concentration data are in excellent agreement.

Conclusions

It appears from these results that the Ferguson principle may be applied to soluble surface-active quaternary ammonium salts when surface concentrations are used and when both microbiological and surface studies are made under identical conditions. It would seem that the mechanism of action of these compounds, although complex, depends primarily on a physical relationship between an external phase and the biophase, e.g., the cytoplasmic membrane.

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Effects of acetylcholine, vagal stimulation and tyramine on the isolated atria of the tortoise

YUNG IN KIM AND PARK CHANG SHIN

In the vagus-atrial preparation of the tortoise (*Amyda japonica*), a cold-blooded animal, acetylcholine and vagal stimulation, in the presence of atropine, produced positive inotropic effects which were inhibited by dichloroisoprenaline or pronethalol. Hexamethonium blocked the excitatory effect of acetylcholine but only slightly inhibited that of vagal stimulation. Tyramine exerted a positive inotropic action in this preparation, but had little effect on atria prepared from reserpine-treated animals. Tyramine was shown to release a substance from the tortoise atria which caused contraction of aortic strips taken from reserpine-treated rabbits, and which appeared to be a catecholamine.

VAGAL stimulation or acetylcholine may produce sympathomimetic effects on the heart or auricle of warm-blooded animals, probably by releasing catecholamines from sites where they are bound (for literature, see Burn & Rand, 1962). However, this effect has not previously been demonstrated in cold-blooded animals. This paper describes the positive inotropic effect of vagal stimulation and of acetylcholine on a vagus-atrial preparation of a cold-blooded animal, the tortoise (*Amyda japonica*). The release of vasoactive material from the same preparation by tyramine was also demonstrated.

Methods

Tortoise vagus-atrial preparation. Tortoises (300–400 g) were killed by severing the cervical spine with a bone cutter and then pithing the spinal cord with a probe. The tortoise was fixed on its back on a board, the plastron was removed and the pericardium cut. The skin covering the neck was cut along the midline and the right vagus nerve was carefully isolated from the right carotid artery and cut at the cranial end. The heart with the right vagus was dissected out and placed in a petri dish containing oxygenated Ringer solution. The ventricle was cut away without injury to the atria. The apex of one atrium was ligated with a thread, and that of the other was clipped with a serffine. The atria were suspended in a 30 ml bath containing oxygenated Frog Ringer solution (NaCl 6·4, KCl 0·3, $CaCl_2$ 0·18, $MgCl_2$ 0·01, $NaHCO_3$ 0·3, glucose 2·0 g/litre) at 25°. The right vagus nerve was placed on platinum electrodes immersed in the bath fluid. The electrodes were raised to the surface of the Ringer solution when stimulation was applied. The atria thus prepared showed regular and spontaneous contractions at a rate of 18 to 30/min. Contractions of the atria were recorded on a smoked drum by a light lever which magnified the contractions 7 times. The experiments were made during the summers of 1962 and 1963.

Rabbit aortic strip. This was prepared by the method of Furchgott (1960), 24–48 hr after reserpine treatment (2-4 mg/kg, intravenously).

From Chon-nam University Medical School, Kwangju, Chollanam-do, Republic of Korea.

The aortic strips were suspended in a 10 ml bath containing oxygenated Krebs bicarbonate solution at 37°.

Guinea-pig ileum. A piece of ileum, 4 cm long, was suspended in Tyrode solution at 29°.

Drugs. All drugs were dissolved in 0·6% w/v saline solution except noradrenaline which was dissolved in acid saline (pH: about 4·1). Drugs were added to the bath in volumes of 0·5–1·0 ml. The drugs used were acetylcholine bromide, noradrenaline bitartrate, tyramine hydrochloride, atropine sulphate, reserpine phosphate, dichloroisoprenaline, pronethalol, hexamethonium chloride, 5-hydroxytryptamine creatinine sulphate, histamine phosphate, 2-bromolysergic acid diethylamide (BOL-148), mepyramine maleate and EDTA disodium salt.

Results

Experiments with untreated preparations. Vagal stimulation and acetylcholine produced inhibition or arrest of the atrial movement. The threshold stimulus parameters necessary to arrest the movement varied from one preparation to another but usually rectangular pulses of 7 to 10 V strength and of 1 msec duration applied at a frequency of 3 to 6/sec for 10 to 15 sec were adequate for this effect. The minimal inhibitory concentration of acetylcholine ranged from 0·1 to 1 μg/ml.

Noradrenaline produced an increase in contraction height; its effect on the atrial rate was not consistent unless the original rate was distinctly low. The doses of noradrenaline to produce about a 20% increase of the contraction heights were 0·02 to 0·1 μg/ml.

Atropine (5 μg/ml) blocked the inhibitory effects of vagal stimulation and of acetylcholine and in most experiments caused a slight increase in contraction height.

Experiments with atropinized preparations. In the presence of atropine (5 μg/ml) an increase in the frequency and duration of vagal stimulation (25–50/sec for 2 min) produced a positive inotropic effect in most atria (27 out of 31 experiments). The magnitude of this response varied in each preparation but was usually of the order of a 10–20% increase in the contraction height (Fig. 1). The responses of each preparation were

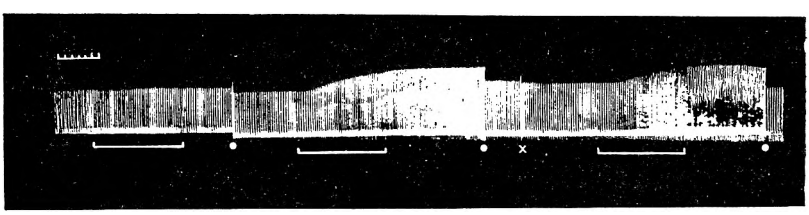

FIG. 1. Effects of vagal stimulation on atropinised isolated vagus-atrial preparation of tortoise. The horizontal lines mark the duration of vagal stimulation (5/sec in the left-hand panel and 25/sec in the other two panels). At the white dots the bath fluid was changed and the kymograph was stopped for 15 min. At x the kymograph was stopped for 5 min. Time marker: 10 sec.

almost consistent when stimulation was repeated at intervals of 15 min for a period of 3 to 4 hr. The chronotropic effect of vagal stimulation was not distinct unless the original atrial rate was low.

Acetylcholine (1 μg/ml) added to the atropinised atria was without inhibitory effect but produced a slight augmentation in some preparations. In 32 out of 37 experiments, an increase in the dose of acetylcholine to 10–100 μg/ml elicited a positive inotropic effect, which was usually of the order of a 20–40% increase in the contraction height. Constant responses were obtained when acetylcholine was added at intervals of 20 to 30 min (Fig. 2).

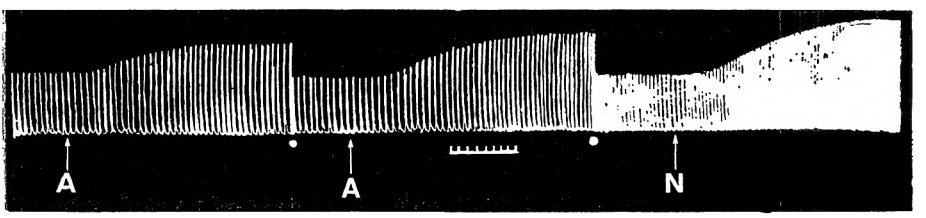

FIG. 2. Effects of acetylcholine and noradrenaline on atropinised isolated atrial preparation of tortoise. At A, 10 μg/ml of acetylcholine and at N, 0·05 μg/ml of noradrenaline were added. At the white dots the bath fluid was changed and the kymograph was stopped for 20 min. Time marker: 10 sec.

Treatment of the atropinised preparation with dichloroisoprenaline (5–10 μg/ml) or pronethalol (1–5 μg/ml), which have been shown to block the action of noradrenaline in the heart (Moran & Perkins, 1958; Black & Stephenson, 1962), markedly reduced or abolished the augmentatory effects of acetylcholine and of noradrenaline. The effect of vagal stimulation was always reduced by both substances but was never completely abolished; i.e. in the presence of dichloroisoprenaline the increase in the contraction height was 13% (average of 5 atria) of the control increase; pronethalol reduced it to 34% (average of 4 atria) of the control increase. These effects of dichloroisoprenaline and pronethalol were reversible.

Treatment of the atria with hexamethonium (100–500 μg/ml) in the presence of atropine abolished the augmentatory effect of acetylcholine, but only slightly inhibited that of vagal stimulation; i.e. in the presence of hexamethonium the increase of the contraction height by vagal stimulation was 79% (average of 6 atria) of the control increase. The hexamethonium effect was reversible. The effect of noradrenaline was not affected by this dose of hexamethonium.

Experiments with preparations from reserpine-treated animals. In some experiments the vagus-atrial preparations were taken from reserpine-treated tortoises (0·1 mg of reserpine phosphate/100 g body weight intraperitoneally 48 to 72 hr previously). The contraction amplitude of atria from reserpine-treated animals (average of 9 atria: 13·3 $\pm$ 2·7 mm) was significantly shorter ($P < 0{\cdot}01$) than that of atria from control animals treated with saline instead of reserpine (average of 10 atria: 26·4 $\pm$ 2·4 mm) when compared under the same conditions (Fig. 3).

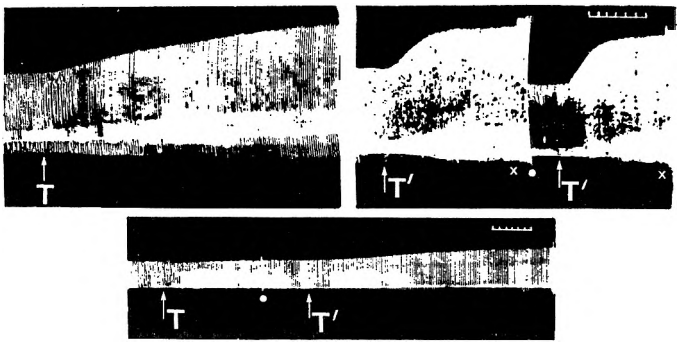

FIG. 3. Effects of tyramine on isolated atrial preparation of tortoise. At T, 2 μg/ml and at T', 20 μg/ml of tyramine were added. The lower panel is a record of a preparation from a reserpine-treated tortoise; the upper panels are records from non-reserpinised preparations. Each panel was taken from a different experiment. At the white dots the bath fluid was changed and the kymograph stopped for 30 min. At x the kymograph was stopped for 2 min. Time marker: 10 sec.

In experiments on nine reserpine-treated atria, five responded to vagal stimulation and 2 responded to acetylcholine with a positive inotropic effect. In the controls, which were given saline instead of reserpine, 14 out of 16 responded to vagal stimulation and 11 out of 15 responded to acetylcholine. The difference was not significant in the case of vagal stimulation but was so with acetylcholine ($P < 0{\cdot}05$).

Response to tyramine. Tyramine (above 2 μg/ml) exerted a distinct augmentatory effect on the tortoise atria. The responses remained fairly constant when tyramine was added every 30 min for 3 to 4 hr. Contrarily, when the same dose of tyramine was added to the atria obtained from reserpine-treated tortoises, the excitatory effect was very slight in all cases (Fig. 3).

Release of vasoactive substance from the tortoise atria by tyramine. In this series of experiments, the tortoise atrial preparations were suspended in 10 ml Ringer solution. Before addition of tyramine the fluid in the organ bath was changed several times and EDTA (10 μg/ml) was added to prevent oxidation of any catecholamine liberated (Crout, Muskus & Trendelenburg, 1962). This dose of EDTA did not affect the atrial movement. The preparation was exposed to tyramine (20 μg/ml) for 10 min. The fluid of the bath was then removed, warmed to 37° and applied to an aortic spiral strip from a reserpinised rabbit.

Tyramine (20 μg/ml) alone or added to bath fluid from an atrial preparation which had not been exposed to tyramine, had little effect on the aortic strip. Neither addition of EDTA nor exchanging the Krebs bicarbonate solution with the hypotonic Frog Ringer caused contraction of the aortic strip.

The application of the bath fluid from an atrial preparation which had been stimulated by tyramine (20 μg/ml) for 10 min always shortened the aortic strip (Fig. 4). When an atrial preparation was exposed to tyramine two or three times at intervals of 50 to 60 min, each sample of bath fluid

contracted the aortic strip. The magnitudes of the contractions of the aortic strip produced by the vasoactive material in the bath fluid (15 applications of tyramine were made to 6 preparations) were almost constant and about equivalent to those produced by 0·005–0·001 μg/ml of noradrenaline.

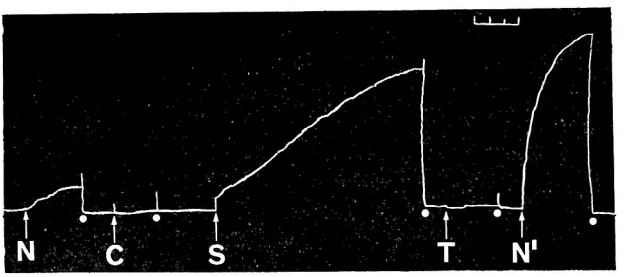

FIG. 4. Responses of an aortic strip from a reserpinised rabbit. At N, 0·001 μg/ml of noradrenaline, at N', 0·005 μg/ml of noradrenaline and at T, 20 μg/ml of tyramine were added. At C, the bath fluid was exchanged for warmed Ringer solution containing EDTA and which had bathed a tortoise atrial preparation for 10 min in the absence of tyramine. At S, the bath fluid was exchanged for Ringer solution containing EDTA and which had bathed a tortoise preparation for 10 min in the presence of 20 μg/ml of tyramine. Note that this concentration of tyramine itself (T) was without effect. At the white dots the bath fluid was exchanged for fresh Krebs solution at 37°. Time marker: 1 min.

In the presence of BOL-148 (0·05 μg/ml), which abolished the vasoactive property of 5-hydroxytryptamine (0·02 μg/ml) but had little effect on that of noradrenaline, the contractions of the aortic strip produced by the bath fluid from the atrial preparations stimulated by tyramine were unaltered. Mepyramine (0·01 μg/ml), which abolished contractions produced by histamine (0·01 μg/ml) but not those produced by noradrenaline, was also without effect on contractions produced by the vasoactive substance in the bath fluid.

Guinea-pig ileum which is known to be highly sensitive to histamine, 5-hydroxytryptamine and vasoactive polypeptides did not contract in response to the bath fluid from atrial preparations stimulated by tyramine.

Discussion

The experiments showed that acetylcholine or vagal stimulation may exert positive inotropic effects in atropinised atria from the tortoise, as they do in those of warm-blooded animals. Inhibition of the inotropic effect by dichloroisoprenaline or pronethalol suggests participation of adrenoceptive receptors on the atrial tissues in producing this effect. Thus the finding is against the opinion of Hashimoto, Kumakura & Hashimoto (1963) that differentiation of adrenergic and cholinergic receptors is incomplete in phylogenetically underdeveloped vertebrates.

The significant decrease in the number of preparations showing the positive inotropic effect in response to acetylcholine after reserpine treatment suggests that liberation of catecholamines is responsible for the

effect. As in other species, the ability of acetylcholine to release catecholamines, but not the action of noradrenaline itself, was blocked by hexamethonium. Acetylcholine may cause the release of catecholamine from chromaffin cells or it may stimulate adrenergic nerve terminals as suggested by Pathak (1958) for the frog heart.

It seemed that the mechanism underlying the inotropic action of vagal stimulation was different from that of applied acetylcholine, as reserpine pretreatment or hexamethonium did not much affect the response to vagal stimulation. However, the difference may be quantitative rather than qualitative; larger doses of reserpine and hexamethonium may be necessary for inhibition of the responses to vagal stimulation.

The present study supplied direct evidence that tyramine liberates vasoconstrictor material from the atria of the cold-blooded tortoise, as it does in warm-blooded animals (Hall, 1963). The control experiments with specific blocking agents suggested that the vasoactive material was a catecholamine. Reserpinization decreased the contraction height of the atria of the tortoise, an effect which has also been described for warm blooded animals.

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Simplified gas chromatographic analysis of pesticides from blood

N. C. JAIN, C. R. FONTAN AND P. L. KIRK

A rapid method for the extraction and identification of 23 pesticides present in blood is described. The pesticides are extracted from blood with a mixture of acetone and ethyl ether in equal volumes. The extracts are evaporated to dryness, the residue dissolved in a known quantity of hexane and an aliquot injected directly into the gas chromatograph equipped with an electron capture detector. No purification of the acetone-ether extract is required. A glass column packed with 5% SE 52 on hexamethyldisilizane-treated chromosorb W 60/80 is used at a temperature of 190°. The method was successfully applied to blood from laboratory animals to which acute doses of six of the most common pesticides were given orally. The quantity of pesticides found is reported.

SYSTEMATIC schemes for the isolation and identification of toxic substances are largely confined to conventional poisons. With the advent of many new compounds having toxic properties, the deficiencies of the classical procedures have been made more obvious. This is especially true of the group of pesticides which includes chlorinated hydrocarbons and organophosphorus compounds. Symptoms associated with these have been treated as chronic rather than acute effects, and analysis has largely been directed towards the control of pesticide residues on foodstuffs.

Acute poisoning from these materials is increasing and the toxicologist needs to be able to identify rapidly and with certainty the presence in physiological materials of acutely toxic quantities of such materials. The quantities of pesticides in such cases are so small, and the conventional tests so insensitive and non-specific, that these poisons may readily be overlooked. It is therefore essential to make available simple and rapid methods of extraction to be used with detection methods to ensure that toxic material is not overlooked. Also the procedure should not require extensive clean up of the extracts before it can be applied.

Various papers have been published (Clifford, 1947; Schechter, Pogorelskin & Haller, 1947; Fairing & Warrington, 1950; Jones & Riddick, 1951, 1952; Krzeminski & Landmann, 1963) describing the isolation of minute amounts of pesticides from biological specimens by means that are usually complex and tedious. Extensive cleanup is required, and is accomplished for example by hexane: aceto-nitrile partitioning (Jones & Riddick, 1952; McKinley & Savary, 1962), paper chromatography (Mitchell, 1957; Müller, Ernst & Schock, 1957; MacRae & McKinley, 1961), thin layer chromatography (Walker and Beroza, 1963), silicic acid (Moats, 1962) and florisil column chromatography (Moddes, 1961; Moats, 1963). A rapid and simple general method for isolating and detecting a group of pesticides from blood is now described.

The method employs the acetone-ether extraction procedure for the gas chromatographic analysis of barbiturates described by Jain, Fontan

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& Kirk (1964). Because of the small amount of impurities present in blood, as compared to tissue, blood was chosen for study.

The method does not require cleanup of the extract, contamination of the detector is negligible, and low levels of organophosphorus compounds and chlorinated hydrocarbons can be detected. Most of the glassware used is disposable, which also eliminates much trouble and inconvenience.

Experimental

MATERIALS

The Hy-Fi gas chromatograph, Aerograph Model 600 (Wilkens Instrument and Research, Inc., Walnut Creek, California), with an electron capture detector, and the Leeds and Northrup Speedomax H, zero to 1 mV recorder, model S, were used. The chromatographic column was a spiral Pyrex glass tube of 0·125 inch outside diameter, 0·070 inch inside diameter, and 4½ feet in length. The solid support material was acid washed Chromosorb W 60 to 80 mesh which was treated with hexamethyldisilizane. This was coated with 5% (w/w) SE 52*.

The stationary phase was applied by forming a slurry of SE 52 in chloroform with the solid support and evaporating the excess solvent rapidly on a steam-bath under vacuum. When dry, the material was packed in the glass column by applying vacuum to one end and gently tapping the column mechanically. Occasionally, nitrogen pressure was applied at the other end to ensure uniform and tight packing.

Standard solutions. Pesticide solutions for use in testing recovery were prepared by dissolving the appropriate quantity of various compounds as listed under Results, in 90 to 100% purity in hexane (Reagent A.C.S.) to give a concentration of 1 mg/ml. These solutions were diluted further as needed; e.g., lindane was injected at concentrations down to 0·2 ng/μl. Standard solutions were chromatographed to determine the retention times of the pesticides studied.

Extraction solvent. The extraction solvent was made by mixing equal volumes of acetone and ether (A.R.). This was used to extract the pesticides from blood samples in all instances before chromatographing the material. Samples examined included 16 compounds added in known quantities to human blood containing EDTA as anticoagulant, and blood from 17 rats which had been given one of six different pesticides as detailed under Results, and blood from 3 rats used as controls.

The rats were healthy females of the Long Evans strain, 35 to 40 days old, weighing between 106 and 137 g; they were separately caged and were reared on White Diet.†

PROCEDURES

All pesticides, both standard solutions and extracts, were dissolved in hexane for gas chromatography. The operating conditions were as

*Phenylmethyl silicone.

†White Diet consists of: ground whole wheat 67·5, casein, tech. 15·0, skim milk powder 7·5, sodium chloride 0·75, calcium carbonate 1·5, melted fat 6·75, fish oil 1·0% and KI solution 0·9 mg iodine/g diet (in solution).

follows: Oven temperature, 190°, injector temperature, 230°, and flow rate of carrier gas (nitrogen), 70 ml. Input impedance was 10^7 ohm and output sensitivity 1X, corresponding to $3{\cdot}3 \times 10^{-9}$ A for full scale recorder deflection. Detector voltage was −90 V. All samples, dissolved in hexane, were injected into the sampling port with a Hamilton syringe of suitable capacity.

Extraction. All blood samples were extracted and gas chromatographed to observe the normal background response and the recovery of pesticide. For convenience, 500 μl of each blood sample was ordinarily taken in a 1/2 dram shell vial. A quantity of mixed solvent (acetone-ether) was added and the mixture stirred for a few sec with a melting-point capillary of 1·3 to 1·5 mm diameter with sealed end, after which the clear supernatant was transferred to another 2 dram shell vial with a Pasteur pipette. This process was repeated three times, fresh solvent being used each time and all supernatants being combined in the second shell vial. The combined extracts were evaporated to dryness at room temperature with a fine jet of air impinging on the surface of the sample. This was conveniently achieved by using a bell jar with a side arm connected to the vacuum line. A glass capillary was mounted through the top opening of the jar to provide the air flow.

The residue was dissolved in 500 μl of hexane, the suspended material was allowed to settle for a few sec, and 1 μl of the clear supernatant was injected into the gas chromatograph. To test the applicability of the method to very small samples of blood, the quantity of sample was reduced to 10 μl for blood from rats fed lindane in LD50 doses (150 mg/kg). The residues of the 10 μl samples, after extraction and evaporation, were dissolved in 100 μl of hexane, and 1 μl of this was chromatographed in the usual manner.

Animals. Pesticides were administered in olive oil solution by stomach tube to 17 animals after they had fasted for 24 hr. One control rat received 1·5 ml of olive oil, and two were given nothing. Pesticides were administered at the LD50 dosage quoted by Negherbon (1959). At varying periods from 0·4 to 9 hr, blood was collected from the descending aorta while the animals were under ether anaesthesia. After as much blood as possible was withdrawn into a heparinised syringe, the rats were bled to death by severing the aorta. The samples were transferred to clean bottles and stored under refrigeration until required for extraction.

Results and discussion

The relative retention values of 23 pesticides, 10 of them organophosphates, 12 of them chlorinated hydrocarbons, and 1 nitro-compound (Morocide) were determined relative to lindane. The reproducibility was good; shorter retention times could be reproduced accurately and the longer ones with errors of 5–7%, providing other conditions remained constant. These values are shown in Table 1, along with the approximate recovery values of 16 of the materials which were added to blood at a level of 1 ppm, extracted, and subjected to gas chromatography.

TABLE 1. RELATIVE RETENTION VALUES AND RECOVERY OF PESTICIDES

Pesticides	Relative retention values	Approximate recovery from blood %*
Aldrin	2·05	100
Chlordane	1·7, 3·3, 3·6	—
DDT (or TDE)	4·7, 6·2	—
DDT	4·3, 6·1, 7·9	—
p-p' DDT	7·9	85–90
Diazinon	1·15	—
Dibrom	0·15	—
Dieldrin	4·5	90–95
Di-syston	1·2	85
Endrin	5·1	100
EPN	12·5	100
Ethion	6·6	95
Heptachlor	1·6, 3·3	100
Heptachlor epoxide	2·8	90
Lindane	1·0	100
Malathion	2·7	95
Methoxychlor	12·9	85
Methyl trithion	5·4	90
Morocide	5·8	75
Parathion	2·4	92–95
PCNB tetrachlor	0·95	—
Thimet	0·8	—
Trithion	7·6	90

Conditions: Oven temperature 190°; injector 230°.
4½ ft. glass column packed with 5% SE 52 on hexamethyldisilizane-treated Chromosorb W, 60–80 mesh.
Flow rate of carrier gas (nitrogen) 70 ml/min.

* The % recoveries reported are calculated to the technical grades of pesticides used as standards.

Certain members of similar groups which were also chromatographed were not extracted, since their behaviour essentially should be identical with those studied. Recoveries were calculated, as were the concentrations of pesticides in the bloods of rats (Table 2), by peak areas based on

TABLE 2. DETAILS OF ANIMAL EXERIMENTS

No. of rats	Pesticides	LD50 dosage mg/kg	Time between dosing and drawing blood (hr)	ppm of pesticide found in the whole blood
3	DDT	200	4	DDE 50 *o-p'* 7 *p-p'* 6–7
1	Dieldrin	50	4	0·67
1	Dieldrin	75	9	2·2
1	Endrin	5	4	None
1	Endrin	10	4	Trace
1	Endrin	20	4	Trace
1	Endrin	50	9	1
2	Lindane	150	4	10
3	Malathion	1000	4	6·6
3	Parathion	5	0·4	3·3

a simple triangulation procedure. To take into account the change in the detector response with time, standards were run frequently, preferably with each injection. During the time of this study such detector response did not vary significantly. Both unknown and known samples were chromatographed in about the same concentration to maintain the linearity of response and to facilitate the quantitative work.

To interpret the results of pesticide extracts from blood, it was essential to determine what effect, if any, normal blood would exert when extracted and the material chromatographed. With the extreme sensitivity of the

electron capture detector for certain types of compounds, it was conceivable that some normal blood constituents might be extracted and recorded by the instrument. Similar considerations also applied to possible impurities present in the solvents. Results showed that no significant response was observed from normal blood, and by using A.R. grade solvents the response from solvent residue was also minimised.

Certain additional experimental points were found to be of significance. Initially, the extracts were evaporated at raised temperature to dry them rapidly, but this was found to lead to loss of most of the materials studied, and was abandoned in favour of the slower procedure described, in which loss did not occur. This procedure was much faster than evaporating spontaneously in the air, which was also tested and gave equivalent results. Another point of interest was the requisite number of extractions needed to obtain quantitative recovery. Several experiments were run with three, as opposed to six, extractions. No significant differences were found, and the procedure of using three extractions was adopted.

Female rats were administered LD50 doses of six of the most commonly used pesticides, two organophosphorus compounds, and four chlorinated hydrocarbons, as described. The general results of these experiments are shown in Table 2. One of three rats which was given parathion showed severe tremors and convulsions within 20 to 25 min of ingestion. One of three that received malathion showed violent convulsions 3 hr after feeding, the other two showed no symptoms up to 4 hr after dosing. No physiological effects were noted with endrin, and the blood contained minimal quantities of the material, even after 9 hr. All other pesticides were detected in significant amounts in the blood at levels insufficient to produce death. The failure to obtain an approximate 50% death rate may be because of the species used, to the small number of animals, and to the fact that technical rather than purified grades of pesticide were used.

Blood was examined rather than fat which is usual for chlorinated hydrocarbons, because we were interested in acute rather than in chronic poisoning. The limited number of experiments did not allow determination of the time at which each pesticide reached maximum concentration in blood. However, in acute poisoning, time is more vital than maximum analytical response, and the times used are suitable for rapid screening tests of affected persons.

The amount of blood analysed, 0·5 ml, was chosen for convenience, and would be readily available in an acute poisoning; with lindane much smaller samples from the finger or ear lobe, were found sufficient. Only a small part of each sample extracted was injected into the gas chromatograph and therefore much smaller quantities of blood could suffice. It would also make experimentation with animals smaller than rats practicable.

Dale, Gaines, Hayes & Pearce (1963) showed that DDT occurs in the plasma of rats fed with this material in peanut oil at the rate of 150 mg/kg of body weight. No similar data have been noted for the organophosphorus pesticides. Our findings demonstrate that the pesticides are to be found in blood in significant quantity in acute poisoning.

The analytical procedure has some advantages over more complex methods. The solvent system is effective, may be used rapidly and is applicable to a wide variety of toxic substances other than pesticides. With blood there is no significant blank readings nor is the electron capture detector disturbed by accumulation of materials. SE52 on a support of hexamethyldisilizane-treated Chromosorb W 60/80 caused little contamination of the detector and the solid support showed little adsorption capacity compared with other supports tested.

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Diuretic effect of some adrenocortical steroids in the rat

P. F. D'ARCY AND E. M. HOWARD

Cortisone, hydrocortisone and some of their Δ^1- and fluorinated analogues have a pronounced diuretic effect in the rat; this is accompanied by increased urinary excretion of both Na^+ and K^+. Diuresis is maximal some 4 hr after oral administration, and in acute studies, the diuretic potency of some of the newer corticosteroids exceeds 100 times that of chlorothiazide.

PROLONGED administration of some adrenocortical steroids to rats causes retardation of body growth (D'Arcy & Howard, 1958a, b, 1961a, 1962). Although it was certain that this effect was due to the action of these steroids on protein catabolism and in particular on the formation of carbohydrate from protein, it was observed that urine excretion was increased, and it was thought that tissue dehydration might be an influencing factor in the growth retardation.

Fielder, Hoff, Thomas, Tolksdorf, Perlman & Cronin (1959) and Wozniak, Paino, Ringler & Roepke (1960) have shown that triamcinolone, administered acutely or sub-acutely to dogs, produces a diuresis and a significant loss of body weight. Several clinicians (Bilka & Melby, 1958; Curd & Spurr, 1958; Feinberg, Feinberg & Fisherman, 1958; Freyberg, Berntsen & Hellman, 1958) have also observed natriuresis and diuresis among the untoward effects following the use of some newer corticosteroids in man.

We have assessed the effect of acute and sub-acute doses of various corticoids on urinary output in the rat and have determined whether this influenced the loss of body weight.

Experimental

Male albino rats, 120–150 g weight, of the Tuck strain were used. They were housed in a thermostatically controlled room at 68–70° F and maintained on a cube diet; tap water was provided *ad lib.* Adrenocortical steroids and chlorothiazide were administered orally in a dose volume of 0·5 ml/100 g weight, to groups of 4 or 5 rats. Control groups were given the diluent alone (5% gum acacia in distilled water) in similar dose volumes. Deoxycortone acetate and a long-acting preparation of adrenocorticotrophic hormone (Cortrophin-ZN) were injected intramuscularly, half of each dose into each thigh muscle; deoxycortone acetate was dissolved in arachis oil and Cortrophin-ZN diluted in saline. Controls were injected with similar volumes of arachis oil or saline intramuscularly.

Urine was collected according to Brittain (1959), and urine excretion was measured over a period of 8 hr during which the animals were deprived of both food and water. Rats were conditioned to the urine collection apparatus before their use in diuretic tests (D'Arcy, 1962). The levels of Na^+ and K^+in the urine were estimated using a flame photometer.

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Results

EFFECT OF CORTICOSTEROIDS ON URINE OUTPUT

The oral administration of cortisone acetate and hydrocortisone acetate (0·63, 1·25, 2·5 and 10 mg/100 g), prednisone (0·31, 0·63 and 2·5 mg/100 g) and prednisolone acetate (0·16, 0·31, 0·63, 1·25 and 2·5 mg/100 g) produced a demonstrable increase in urine output. Urine excretion was maximal some 4 hr after dosage for the steroids examined; Fig. 1 illustrates the diuretic effect of cortisone acetate.

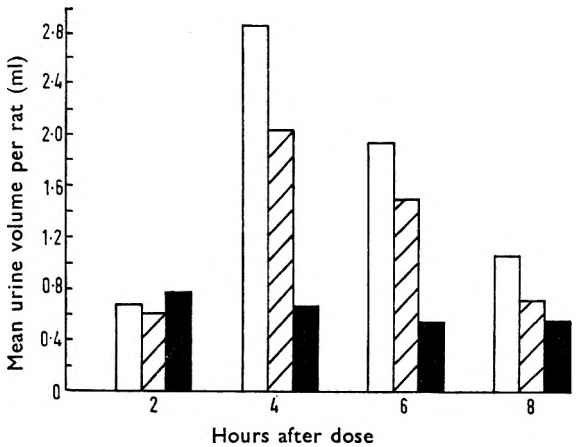

FIG. 1. Mean urine excretion per rat at 2 hr intervals after oral dosage with cortisone acetate. Each dose of the steroid was given in a volume of 0·5 ml/100 g body weight to 2 groups of 4 rats; control rats received a similar dose of the vehicle. Open columns: cortisone acetate 10 mg/100 g. Hatched columns: cortisone acetate 2·5 mg/100 g. Solid columns: controls.

The diuretic effect of these steroids increased with increase in dosage; in Fig. 2 the degree of diuresis has been expressed by a "Diuretic Index", which is the ratio of urine volume from treated animals to that of the controls. Chlorothiazide (0·63–20 mg/100 g) was included for comparison.

All these steroids showed greater diuretic activity than chlorothiazide in total urine excretion for 8 hr after dosage. 9α-Fluorohydrocortisone (fludrocortisone) acetate (0·31–5·0 mg/100 g), Δ^1-9α-fluorohydrocortisone (0·16–1·25 mg/100 g) and 9α-fluoro-16α-hydroxyprednisolone (triamcinolone) (0·02–1·25 mg/100 g) had an even greater effect on urine excretion. Deoxycortone acetate (1·0–25 mg/100 g) produced some diuresis but the effect was much less than that produced by chlorothiazide or by any of the other steroids; similarly Cortrophin-ZN had diuretic activity when injected intramuscularly at doses of 1·25–10 mg/100 g, although it was less potent than chlorothiazide. This response, although weak, indicates that stimulation of the output of endogenous adrenal steroids also promotes diuresis. These results are summarised in Table 1, and an approximation of the diuretic potency of the steroids relative to chlorothiazide under the conditions of the test has been made in Table 2.

TABLE 1. THE DIURETIC EFFECT OF SOME ADRENOCORTICAL STEROIDS, CORTICOTROPHIN AND CHLOROTHIAZIDE

Steroid or other agents	Dose mg/100 g/ orally	No. of rats	Mean urine volume per test rat per 8 hr (ml)	Mean urine volume per control rat per 8 hr (ml)	Diuretic index
Cortisone acetate	10	8	6·55	2·56	2·6
" "	2·5	8	4·84	2·56	1·9
" "	1·25	8	3·45	1·76	2·0
" "	0·63	8	3·13	1·76	1·8
Hydrocortisone acetate	10	5	6·98	2·49	2·8
" "	2·5	5	6·26	2·49	2·5
" "	1·25	10	7·57	3·35	2·3
" "	0·63	10	5·06	3·35	1·5
Prednisone	2·5	4	6·33	1·90	3·3
"	0·63	9	4·34	1·65	2·6
"	0·31	5	1·58	1·45	1·1
"	0·16	5	1·30	1·45	0·9
Prednisolone acetate	2·5	4	6·73	2·54	2·6
" "	1·25	4	6·70	2·54	2·6
" "	0·63	9	5·83	2·45	2·4
" "	0·31	9	5·30	2·45	2·2
" "	0·16	5	3·04	2·38	1·3
" "	0·08	5	3·22	2·38	1·4
Fludrocortisone acetate	5·0	4	5·88	1·19	4·9
"	1·25	4	5·53	1·19	4·6
"	0·63	5	5·42	1·67	3·2
"	0·31	4	2·43	1·19	2·0
"	0·16	5	4·14	1·67	2·5
Δ^1-9α-Fluorohydrocortisone acetate	2·5	4	6·50	1·90	3·4
"	1·25	5	6·68	1·23	5·4
"	0·63	9	5·98	1·52	3·9
"	0·31	5	5·00	1·23	4·1
"	0·16	5	4·02	1·23	3·3
Triamcinolone	1·25	5	5·67	1·56	3·6
"	0·63	5	5·68	1·56	3·6
"	0·31	5	5·04	1·56	3·2
"	0·16	5	5·15	1·35	3·8
"	0·08	10	4·59	1·82	2·5
"	0·04	10	2·90	2·33	1·3
"	0·02	10	2·86	2·33	1·2
"	0·01	5	2·35	2·35	1·0
Deoxycortone acetate	25*	4	2·01	1·04	1·9
"	10*	8	2·81	2·04	1·4
"	1·0*	4	2·62	2·90	0·9
Cortrophin-ZN	10* units	8	4·20	2·06	2·0
"	5·0* units	8	3·60	2·45	1·5
"	2·5* units	8	4·23	2·60	1·6
"	1·25* units	8	3·21	2·40	1·3
Chlorothiazide	20	15	3·77	1·63	2·4
"	5·0	15	3·09	1·63	1·9
"	2·5	10	4·37	2·39	1·8
"	1·25	10	3·85	2·10	1·8
"	0·63	15	3·31	2·40	1·4

* Intramuscularly

TABLE 2. APPROXIMATE RELATIVE DIURETIC ACTIVITY OF SOME ADRENOCORTICAL STEROIDS, CORTICOTROPHIN AND CHLOROTHIAZIDE

Diuretic agent and route	Relative diuretic activity
Chlorothiazide (oral)	1
Cortisone acetate (oral)	1–2
Hydrocortisone acetate (oral)	4–8
Prednisone (oral)	32–64
Prednisolone acetate (oral)	32–64
Fludrocortisone acetate (oral)	64
Δ^1-9α-Fluorohydrocortisone acetate (oral)	>128
Triamcinolone (oral)	>128
Deoxycortone acetate (i.m.)	<1
Cortrophin-ZN (i.m.)*	<1

* Compared as units of Cortrophin-ZN against mg of chlorothiazide.

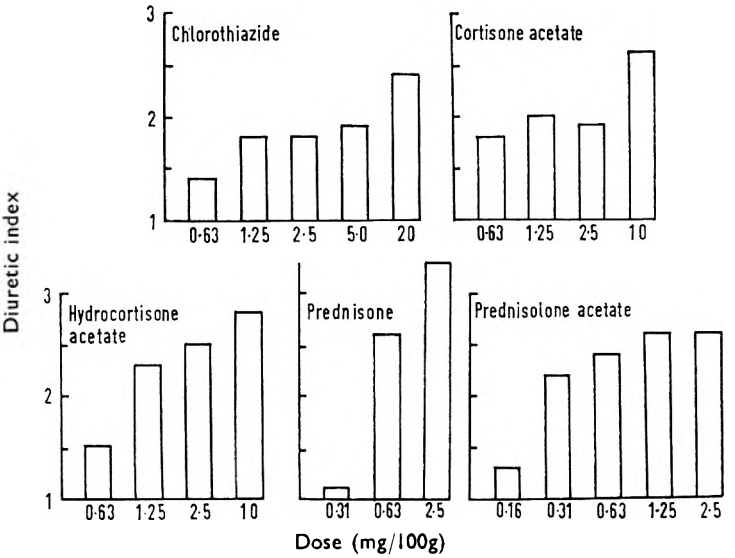

FIG. 2. The effect of oral dosage with chlorothiazide and some corticosteroids on urine excretion in groups of 4 or 5 rats. Urine was collected during 8 hr after dosage. The Diuretic Index is the ratio of urine volume from treated animals to that of the controls.

EFFECT OF CORTICOSTEROIDS ON ELECTROLYTE EXCRETION

The Na^+ and K^+ levels in the urine were measured routinely. These results (Table 3), relate urine output to the concentration of Na^+ and K^+ in the urine, and to the total Na^+ and K^+ excretion per rat during the 8 hr

TABLE 3. THE EFFECT OF ORAL DOSAGE OF SOME ADRENOCORTICAL STEROIDS AND CHLOROTHIAZIDE ON THE Na^+ AND K^+ LEVELS IN THE URINE OF RATS

Treatment	Dose mg/100 g	No. of rats	Mean urine volume excreted per rat (ml) during 8 hr	Na^+ conc in urine m-equiv./litre	K^+ conc in urine m-equiv./litre	Total Na^+ excreted per rat (m-equiv.) in 8 hr	Total K^+ excreted per rat (m-equiv.) during 8 hr
Controls	—	131	2·0 s.e. ± 0·2	150·7 s.e. ± 9·8	122·5 s.e. ± 8·6	0·28 s.e. ± 0·02	0·23 s.e. ± 0·01
Hydrocortisone acetate	10	5	7·0	130·4	76·9	0·91	0·54
" "	2·5	5	6·3	143·5	82·1	0·90	0·52
" "	0·63	10	5·0	134·8	87·3	0·70	0·44
Prednisone	2·5	4	6·3	104·3	66·7	0·66	0·42
"	0·63	9	4·3	147·8	79·5	0·64	0·34
"	0·16	5	1·3	243·4	169·3	0·32	0·22
Fludrocortisone acetate	5·0	4	5·9	95·6	110·3	0·56	0·65
" "	1·25	9	5·8	89·2	116·7	0·52	0·68
"	0·31	4	2·4	95·6	148·8	0·23	0·36
Δ^1-9αFluorohydrocortisone acetate	2·5	4	6·5	143·5	59·0	0·93	0·38
" "	0·63	9	6·0	141·3	116·8	0·85	0·70
" "	0·16	5	4·0	130·4	153·9	0·52	0·62
Triamcinolone	1·25	10	5·7	136·9	103·9	0·78	0·59
"	0·31	10	5·0	123·9	101·4	0·62	0·51
"	0·08	10	5·4	108·6	69·3	0·59	0·37
"	0·02	10	3·5	108·6	77·0	0·38	0·27
Chlorothiazide	20	5	3·3	147·8	92·4	0·49	0·30
"	5·0	5	2·3	165·2	84·7	0·38	0·20
"	2·5	5	2·9	165·2	82·1	0·48	0·24

test. Control results have been pooled in the Table, and the mean values (± s.e.) calculated; they represent results from 131 rats in 27 groups.

With all steroids, the Na^+ concentration in the urine decreased; this did not seem to be directly related to the dose of an individual steroid. There was also a decreased concentration of K^+ and this seemed to be related to the dose of the steroid except triamcinolone. When the Na^+ and K^+ values were expressed as total m-equiv. of electrolyte excreted per rat during 8 hr, the Na^+ excretion increased beyond that of the controls and appeared to become greater as the dose of the steroid increased. The K^+ excretion increased although not to levels as high as those of Na^+; this effect was apparently related to the dose of the steroid. With fludrocortisone, the ratio of Na^+ to K^+, whether as concentration in the urine or as total electrolyte excreted per rat during 8 hr was less than one; with all the other steroids, this ratio was greater than one.

Chlorothiazide, in the doses used, had little effect on the Na^+concentration in the urine, although it did raise the total excretion of this ion during 8 hr. The K^+ concentration was slightly lower in the treated than in the control animals although the total amount of K^+ excreted per rat during 8 hr was normal.

EFFECT OF PROLONGED DOSAGE ON DIURESIS AND ON BODY WEIGHT

The effect on diuresis of steroid given orally was assessed over 14 days, and was compared with the effect of chlorothiazide. Fludrocortisone was selected since it was one of the more potent steroids producing diuresis.

Three groups of 5 rats were used; one group received daily oral doses of fludrocortisone acetate (1·25 mg/100 g), the second group were given chlorothiazide (2·5 mg/100 g), and a control group received the diluent alone (5% gum acacia in distilled water). All animals received their daily dosage in a volume of 0·5 ml/100 g; food and water were withheld during the 8 hr test but animals had unlimited access to both on the days on which, although dosed, they were not placed in the urine collection apparatus. Urine volumes per group were recorded on the first day of the experiment and subsequently at 2 or 3 day intervals; weights were also recorded at these times. The effect of continued dosage on urine output and on weight is shown respectively in Figs 3 and 4.

Daily administration of each drug produced a diuretic effect, which showed a gradual decrease during the 14 days. This reduction was more apparent with the steroid than with chlorothiazide. Although towards the end of the experiment, chlorothiazide produced a diuresis similar to that of the steroid, the weight of the two treated groups differed. The rats dosed with the steroid did not maintain a normal rate of growth, whereas those dosed with chlorothiazide continued to gain weight and the mean body weight did not differ significantly from that of the control animals.

In earlier studies (D'Arcy, Brittain & Howard, 1961), in which rats were treated with adrenal steroids (prednisone, 0·63 and 2·5 mg/100 g; Δ^1-9α-fluorohydrocortisone, 0·63 and 2·5 mg/100 g) orally on alternate days over a period of 14 days, there was no decrease in the diuretic effect.

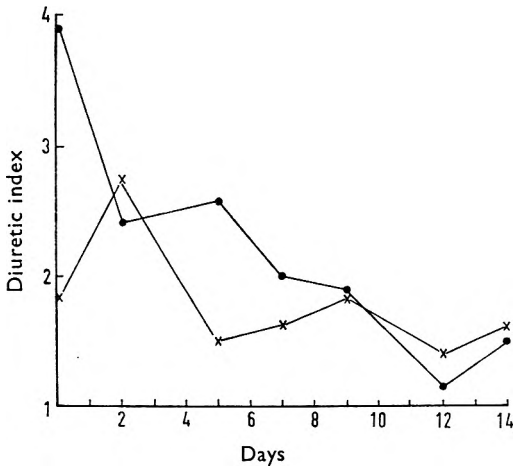

FIG. 3. A comparison between the diuretic effect of chlorothiazide (2·5 mg/100 g orally per day), (×——×) and fludrocortisone acetate (1·25 mg/100 g orally/day), (●——●) during sub-acute (14 days) administration to rats. Groups of 5 rats were used.

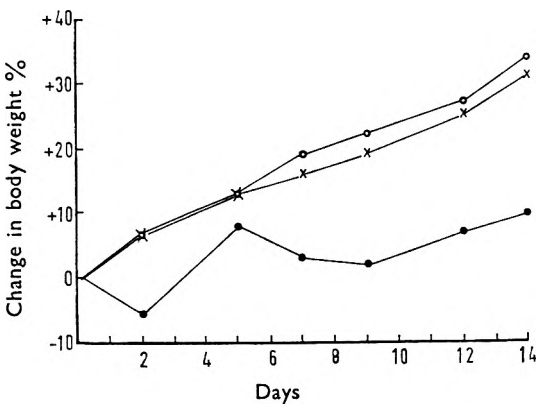

FIG. 4. The effect of daily oral administration of chlorothiazide and fludrocortisone acetate on the body weight of rats. Groups of 5 rats were used; controls (○——○); chlorothiazide (2·5 mg/100 g/day), (×——×); fludrocortisone acetate (1·25 mg/100 g/day), (●——●).

Discussion

Cortisone, hydrocortisone and some of the newer corticosteroids have a pronounced diuretic effect in the rat. This is maximal some 4 hr after oral administration. Acutely, these adrenocortical steroids were more potent diuretics than chlorothiazide; this potency was especially evident with the Δ^1-and fluorinated steroids where maximal activity was at least 100 times (>128) that of chlorothiazide. Cortrophin-ZN, when injected intramuscularly, also had a diuretic effect which, although weak relative to that of the other corticosteroids, suggests that stimulation of the adrenal cortex will also produce diuresis.

Hydrocortisone and its Δ^1- and fluorinated analogues uniformly elicit increases in the urinary excretion of both sodium and potassium; however, the Na^+- and K^+-retaining properties of these steroids are also evident from a comparison of the amounts of the two electrolytes in the urine with the amounts present in urine from control animals. The increase in water excreted is responsible for the overall loss of electrolytes.

The mechanism of the corticosteroid-induced diuresis is uncertain; Heller & Ginsburg (1961) have suggested that the rise in glomerular filtration rate and renal blood flow, which the glucocorticoids usually produce in healthy animals and man, may be a factor of importance. Alternatively, a direct effect on tubular water reabsorption has been both postulated (Jones, 1957) and denied (Skillern, Corcoran & Scherbel, 1956).

Interaction between the antidiuretic hormone (ADH) and the corticosteroid is yet another possibility, and this theoretically could involve a suppression or release of ADH by the supraopticohypophysial system, or an interaction between ADH and the steroid at a common site of action, notably the renal tubule. Initial experiments reported elsewhere (D'Arcy & Howard, 1961b) add support to the suggestion that corticosteroid-ADH interaction may be one of the mechanisms involved in the diuresis, although the findings were not sufficiently extensive to suggest the possible site of this interaction. Prednisolone reduces the antidiuretic effect of vasopressin (Natzschka & Senft, 1959); some corticosteroids interfere with the release of ADH from the neurohypophysis (Gaunt, Lloyd & Chart, 1957; Martini, Pecile & Giuliani, 1960) and they will also promote the disappearance of ADH from the circulation (Ginsburg, 1954).

Changes in weight are the result of a gain or loss of anhydrous tissue and a gain or loss of water; in earlier studies (D'Arcy & Howard, 1960; D'Arcy, Brittain & Howard, 1961) it was suggested that the retardation of body growth in rats dosed subacutely or chronically with adrenocortical steroids might be due partially to dehydration caused by diuresis. In the present work, after daily administration of chlorothiazide and fludrocortisone to rats for 14 days, there was a gradual decrease in the diuretic effect. This rate of decrease was greater with the steroid, which initially was the more potent. After 14 days the diuretic effect of the two drugs was the same. The rats treated with the chlorothiazide daily had a normal increase in weight, whereas rats treated with fludrocortisone showed retardation of growth. It is thus apparent that the failure of rats to gain weight during prolonged corticosteroid dosage is unlikely to be influenced by the diuresis.

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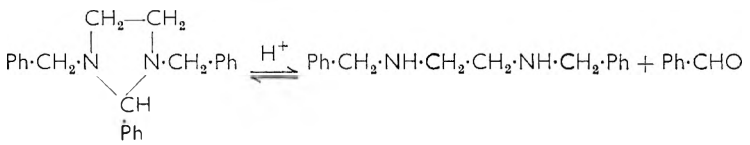
J. Pharm. Pharmacol., 1965, **17**, 376–378 Received January 14, 1965

Estimation of 1,3-dibenzyl-2-phenyltetrahydroimidazole in *NN'*-dibenzylethylenediamine diacetate

A. W. ARCHER

A simple spectrophotometric method is described for the estimation of 1,3-dibenzyl-2-phenyltetrahydroimidazole in *NN'*-dibenzylethylenediamine diacetate. The imidazole is quantitatively hydrolysed by dilute sulphuric acid to benzaldehyde and *NN'*-dibenzylethylenediamine; the benzaldehyde is estimated spectrophotometrically after extraction into n-hexane.

THE diamine, *NN'*-dibenzylethylenediamine (II), is used as its diacetate in the preparation of benzathine penicillin (Szabo, Edwards & Bruce, 1951). The diamine is normally obtained by the reduction of *NN'*-dibenzylidene-ethylenediamine (Van Alphen, 1935; Lob, 1936; Szabo & others, 1951); in the course of this reaction some 1,3-dibenzyl-2-phenyltetrahydroimidazole (I) is produced as a by-product (Szabo & others, 1951). This water-insoluble compound is an undesirable impurity as, unless removed, it will be present in the precipitated benzathine penicillin; it was therefore desirable to be able to determine this compound quantitatively.



(I) (II)

Experimental

Lob (1936) reported that (I) was hydrolysed by acids to benzaldehyde and (II). This reaction has been found to be complete within 1 min at room temperature in the presence of dilute sulphuric acid; the resulting benzaldehyde may be extracted with n-hexane and determined spectrophotometrically; *NN'*dibenzylethylenediamine is retained in the acid layer and does not interfere. A sample of (I) was prepared by the method of Van Alphen (Van Alphen, 1935) as a white, microcrystalline solid (m.p. 99°, quoted melting-point 100°; found: C, 83·6; H, 7·4; N, 8·5; calculated for $C_{23}H_{24}N_2$: C, 84·1; H, 7·4; N, 8·5%) and subjected to hydrolysis as described under Procedure; one extraction with an equal volume of n-hexane was sufficient to remove the benzaldehyde produced; a second extract showed negligible absorption at 241 mμ. The results are shown in Table 1.

The ultraviolet spectrum of the material produced by hydrolysis and extracted by hexane was identical to that of benzaldehyde (Analar grade, British Drug Houses, nominal assay: not less than 99%) λ_{max} = 241 mμ,

From Charles E. Frosst & Co., P.O. Box 247, Montreal, Canada.

$E(1\%, 1\text{ cm}) = 1{,}400$, shoulder at 247 mμ, and different to that of the imidazole, $\lambda_{max} = 248$ mμ, $E(1\%, 1\text{ cm}) = 441$. Hydrolysis and recovery of (I) added to *NN'*dibenzylethylenediamine diacetate were found to be satisfactory, as shown in Table 2.

TABLE 1. HYDROLYSIS OF 1,3-DIBENZYL-2-PHENYLTETRAHYDROIMIDAZOLE

Imidazole, μg	Calculated yield of benzaldehyde, μg	Benzaldehyde found, μg*	Hydrolysis %
50	16·15	16·0	99·4
50	16·15	15·3	95·0
100	32·3	30·7	95·0
100	32·3	30·5	94·4
100	32·3	31·2	96·6
100	32·3	33·1	102·5
100	32·3	31·7	98·1
			Average: 97·3%

* Calculated from $E(1\%, 1\text{ cm})$ of benzaldehyde in n-hexane.

TABLE 2. RECOVERY OF 1,3-DIBENZYL-2-PHENYLTETRAHYDROIMIDAZOLE ADDED TO 200 mg *NN'*-DIBENZYLETHYLENEDIAMINE DIACETATE*

Added μg	Found μg	Recovered μg (corrected for blank)	Recovery %
0	33·0	0	—
50	84·7	51·7	103·4
50	85·1	52·1	104·2
100	132·4	99·4	99·4
100	133·5	100·5	100·5
100	134·3	101·3	101·3
		Average recovery: 101·8%	

* Commercial material recrystallised twice from ethyl acetate.

To avoid very small sample weights for material containing larger quantities of the imidazole, it was necessary to prepare a solution containing about 1 mg of the imidazole and to take a suitable aliquot.

PROCEDURE

Weigh accurately a quantity of sample expected to contain about 1 mg of (I) into a 100 ml volumetric flask and dilute to volume with dilute sulphuric acid (10% v/v). Pipette 10 ml of this solution into a stoppered test-tube (25 ml capacity), add 10 ml n-hexane (spectroscopic grade), shake vigorously for 1 min and allow to separate; measure the extinction of the upper layer in a 1 cm cell at 241 mμ, using hexane as a blank. Determine the $E(1\%, 1\text{ cm})$ of benzaldehyde in n-hexane.

$$\%\ \text{1,3-dibenzyl-2-phenyltetrahydroimidazole} = \frac{100 \times E_{241} \times \text{molecular weight of the imidazole}}{E(1\%, 1\text{ cm}) \text{ of benzaldehyde} \times W \times \text{molecular weight of benzaldehyde}}$$

where W = weight taken in g.

Results

The method was applied to four different batches with the results shown in Table 3.

TABLE 3. RESULTS OBTAINED USING THE PROPOSED METHOD

Batch A	0·0506	0·0509	0·0508	0·0513%	Average 0·0509%
B	0·0369	0·0373	0·0370	0·0371%	0·0370%
C	0·0182	0·0184	0·0177	0·0176%	0·0180%
D	0·0311	0·0319	0·0322	0·0325%	0·0319%

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Effect of body weight changes on the formation of cotton pellet-induced granuloma

G. DIPASQUALE AND A. MELI

Cotton pellet-induced granuloma formation in intact animals is intimately dependent upon body growth and general anabolic processes. Deposition of connective tissue around the pellet continues up to (and probably beyond) the observation period of 90 days after implantation. Anorexic or catabolic agents, or both, or restricted food intake markedly impairs granuloma formation as a result of an impaired body growth. By expressing the amount of granuloma formed in mg/100 g body weight, the true anti-granulomatous properties of a drug can be easily dissociated from those attributable to an impaired body growth. The mechanism by which impairment in body growth affects granuloma formation is discussed.

IT has been reported that the subcutaneous administration of substances which cause marked irritation at the site of injection significantly inhibits the formation of the cotton pellet-induced granuloma (Cygielman & Robson, 1963). These authors conclude that this test is not suitable for the investigation of subcutaneously administered irritating substances. However, this is not the only factor limiting the use of this method. We have repeatedly observed reduced granulomas following the administration of anorexic or catabolic agents or both, or by restricting food intake. To generalise, any appreciable impairment in the normal growth of the animal appears to interfere to a greater or lesser extent with granuloma formation.

In a variety of biological tests, it is common to express the increase or decrease in the weight of certain organs as a percentage of body weight. The application of this conversion factor to the cotton pellet test (mg of granuloma/100 g body weight) makes it possible to dissociate the anti-granulomatous properties of a substance from those attributable to an impairment in body growth.

The object of this paper is to present evidence which indicates the close relationship between body growth and granuloma formation and also to justify the use of the conversion factor.

Experimental

MATERIALS AND METHODS

Male Wistar rats, 160 $\pm$ 10 g, were used. Under light ether anaesthesia, one cotton pellet was introduced subcutaneously on each side of the abdomen through a mid-line incision. The pellets were cut from dental cotton rolls (No. 1 Johnson and Johnson) and paired to a combined dry weight of 70 $\pm$ 1 mg. Before implantation the pellets were individually soaked in 0·5% antibiotic (Combiotic, Pfizer) solution. At the end of the test period, the animals were killed by carbon dioxide asphyxiation. The pellets with the surrounding granuloma were removed, dried to a constant weight at 50° and weighed. The amount in excess of 70 mg represents the weight of the granuloma.

From Warner-Lambert Research Institute, Morris Plains, New Jersey.

Effect of food restriction on granuloma formation. The rats were individually caged and divided into 6 groups of 8 animals each. Group 1 received ground food *ad libitum*, the other groups received a daily amount of ground food equivalent to 10, 8, 6, 4 and 2% of their original body weight. Food restriction was started on the same day as cotton pellet implantation. The animals were killed 10 days later, and the weights of the granulomas were determined.

Effect of body growth on granuloma formation. After cotton pellet implantation, groups of 8 animals were killed at various time intervals up to 90 days, and the weights of the granulomas determined.

Effect of cortisone acetate on granuloma formation. The rats were divided into 5 groups of 14 animals each. Group 1 served as control, whereas the others received varying subcutaneous doses of cortisone acetate. Treatment started on the same day as pellet implantation. The animals were killed 10 days later and the weights of the granulomas were determined.

Results

Effect of food restriction on granuloma formation. The data are shown in Table 1. Compared with controls fed *ad libitum*, a reduced rate of

TABLE 1. EFFECT OF FOOD INTAKE RESTRICTION ON GRANULOMA FORMATION

Amount of food offered as % original body weight	Average body weight in g		Average body weight changes in g	Average weight of granuloma in mg			
	Initial	Final		Absolute values	P	As % body weight	P
Ad libitum (8)*	166	240	+74	97·0 ± 4·6**	—	40·4 ± 2·8**	—
10 (8)*	166	211	+45	88·0 ± 4·6	N.S.	41·7 ± 2·0	N.S.
8 (8)*	169	203	+34	82·0 ± 3·4	<0·025	40·4 ± 1·8	N.S.
6 (8)*	165	165	0	71·0 ± 2·6	<0·001	43·0 ± 1·6	N.S.
4 (8)*	166	135	−31	60·0 ± 2·9	<0·001	44·4 ± 2·3	N.S.
2 (8)*	169	104	−65	42·0 ± 3·3	<0·001	40·4 ± 2·5	N.S.

* Number of animals.
** Standard error.

gain to a severe loss from original body weight occurred as the amount of food made available to the animals was proportionally decreased. If the weight of the granuloma was expressed as an absolute value, a parallel proportional decrease in the weight of the granuloma was observed. However, when the weight of the granuloma was expressed as mg/100 g body weight, no significant difference could be detected between the various groups.

Effect of body growth on granuloma formation. The results are shown in Fig. 1. When the weight of the granuloma is expressed as absolute value, it is apparent from the slope of the two curves that the growth of the granuloma exceeds that of the body up to 30–36 days after implantation and parallels the latter thereafter. This phenomenon is more obvious when the weight of the granuloma is expressed as mg/100 g body weight.

Effect of cortisone acetate on granuloma formation. The results are shown in Table 2. Compared with untreated animals, a reduced rate of

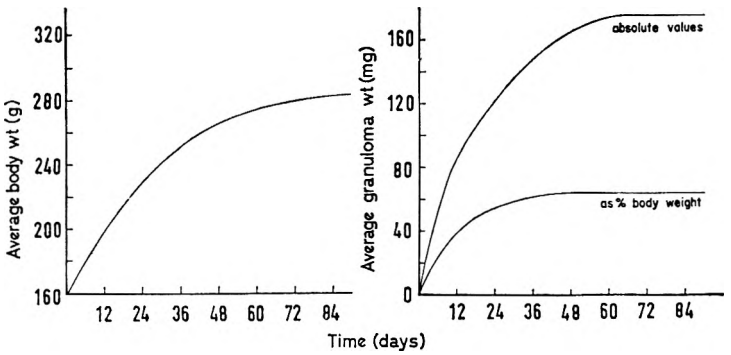

FIG. 1. Effect of body growth on granuloma formation.

TABLE 2. EFFECT OF CORTISONE ACETATE ADMINISTRATION ON GRANULOMA FORMATION

Treatment	Daily dose mg/kg	Route	Average body weight in g		Average body weight changes in g	Average weight of granuloma in mg			
			Initial	Final		Absolute values	P	As % body weight	P
None (14)*	—	—	163	224	+61	98·0 ± 4·3**	—	43·7 ± 1·8**	—
Cortisone Ac. (14)*	5	s.c.	155	210	+55	91·0 ± 6·1	N.S.	43·3 ± 2·5	N.S.
Cortisone Ac. (14)*	10	s.c.	159	194	+35	77·0 ± 4·7	<0·005	39·7 ± 2·2	N.S.
Cortisone Ac. (14)*	20	s.c.	158	175	−17	60·0 ± 3·4	<0·001	34·3 ± 1·7	<0·001
Cortisone Ac. (14)*	40	s.c.	161	156	−5	49·0 ± 1·8	<0·001	31·4 ± 1·1	<0·001

* Number of animals.
** Standard error.

gain to a slight loss from the original body weight occurred in proportion to the amount of subcutaneously administered cortisone acetate. The significant reduction in granuloma formation following the 10 mg/kg dose was probably due to an impairment in body growth since no difference was detectable when the weight of the granuloma was expressed in mg/100 g body weight. At the 20 and 40 mg/kg doses, the reduction in granuloma formation was over and above that which could be attributed to an impairment in body growth. Significant reduction could, therefore, be detected even when the weight of the granuloma was expressed in mg/100 g body weight.

Discussion

The cotton pellet-induced granuloma is a widely used method for assessing anti-inflammatory activity. It seemed, therefore, worthwhile to study one of the aspects, which although not generally taken into consideration, may influence the interpretation of the results.

Our experiments indicate that granuloma formation in intact animals is intimately dependent upon body growth and general anabolic processes. In fact, any impairment in body growth, whether obtained by restricting food intake or by drug administration, considerably slows down the rate of granuloma formation.

The inflammatory reaction produced by the foreign body probably stimulates the rapid deposition of connective tissue around the pellet during the first few days after implantation (Eichhorn & Sniffen, 1964). It is very unlikely that it could be responsible for the further and relatively slower deposition which continues up to and probably beyond 90 days. This latter finding is in disagreement with the observation (Penn & Ashford, 1963) that granuloma formation reaches its peak on the second day and declines thereafter, although the different site of implantation (groin) could have been responsible for such a phenomenon.

Preliminary experiments (unpublished) indicate a decreased effectiveness of anti-inflammatory compounds when the administration of such compounds is proportionally delayed after the time of cotton pellet implantation. It appears that the antigranulomatous effect of cortisone acetate and other anti-inflammatory compounds is twofold: (*a*) direct inhibition of the inflammatory process and (*b*) indirect inhibition caused by the reduced body weight gain or body weight loss. To generalise, if body weight change is not considered, a falsely high anti-inflammatory effect may be attributed to compounds under test.

The decrease in the rate of granuloma formation attributable to an impairment in body growth can be easily dissociated from that produced by drug administration by expressing the amount of granuloma formed in mg/100 g body weight. Our data clearly show that by using this conversion factor, the granuloma inhibitory effect of the lower doses of cortisone acetate are attributable to its well-known anorexic or catabolic activities, or both, which results in a reduced rate of body growth. Only at the higher doses did this compound show an inhibitory effect over and above that which could be attributed to an impairment in body growth.

The mechanism by which restriction in food intake suppresses granuloma formation is not known. Since intact rats were used, an adrenocortical stimulation in response to stress from starvation cannot be excluded. Restricted diets gave responses comparable to high doses of cortisone acetate only when absolute values were used but not when the conversion factor was utilised. If adrenocortical stimulation alone was responsible for this effect, significant reduction should have been evident even when granuloma weight was expressed in mg/100 g body weight, as was seen with the higher doses of cortisone acetate. Another possibility is that inadequate nutrition may directly impair growth and division of fibroblasts in the proliferating granuloma.

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Letters to the Editor

Effect of certain neuromuscular blocking agents on dexamphetamine toxicity in aggregated and isolated mice

SIR,—Since the observation by Gunn & Gurd (1940) that amphetamine is several times more toxic to aggregated than isolated mice, several factors have been found to influence this effect. The major factors are strain and body weight of mice (Chance, 1947; Fink & Larson, 1962), environmental temperature (Höhn & Lasagna, 1960; Askew, 1961; Fink & Larson, 1962; Wolf & George, 1964), noise (Chance, 1946, 1947; Cohen & Lal, 1964), electrical stimuli (Weiss, Laties & Blanton, 1961) and artificially induced aggressive behaviour (Consolo, Garattini & Valzelli, 1965). Greenblatt & Osterberg (1961) attributed the enhanced toxicity of amphetamine aggregation to the enhanced body movements of the mice. Hardinge & Peterson (1963, 1964) reported that amphetamine is as toxic to isolated mice forced to exercise as it was to aggregated mice.

It was reasoned that, if excessive body movements following amphetamine aggregation was a factor responsible for the enhanced lethality, it should be possible to counteract it by pharmacologically induced reduction in the body movements. This aspect was examined by using those neuromuscular blocking agents which act peripherally—(+)-tubocurarine, suxamethonium and gallamine triethiodide—to overcome the excessive movements.

Male albino mice, 25–33 g, were given the drugs or saline injected in 0·01 ml/g body weight. Aggregated mice were placed in plastic cages which allowed 25 cm^2 floor space per mouse, and isolated mice were placed, one mouse per cage with 200 cm^2 floor space. All experiments were made in an air conditioned room at 22–23°.

TABLE 1. INFLUENCE OF NEUROMUSCULAR BLOCKING AGENTS ON DEXAMPHETAMINE TOXICITY IN AGGREGATED AND ISOLATED MICE

Drugs	Aggregation 5 mg/kg dexamphetamine i.p. 10 min later		Isolation 10 mg/kg dexamphetamine i.p. 30 min later	
	No. dead/No. used	% Mortality	No. dead/No. used	% Mortality
Control (Saline)	18/36	50	2/20	10
Tubocurarine 400 µg/kg, s.c. ..	6/12	50	2/10	20
Suxamethonium 2 mg/kg, s.c. ..	7/12	66	1/10	10
Gallamine triethiodide 5 mg/kg, s.c.	5/12	41	0/10	0

From the results of Table 1 it is seen that tubocurarine suxamethonium and gallamine triethiodide do not appreciably alter the lethality of mice in amphetamine aggregation. These results do not, therefore, lend support to the view that reduction in motor activity leads to a decrease in amphetamine aggregation toxicity.

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April 3, 1965

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The spirally cut tracheal strip preparation

SIR,—Castillo & DeBeer (1947) described the tracheal chain preparation for investigating the *in vitro* action of drugs on the tracheal muscle of small laboratory animals. Modifications of this method have since been introduced by Akcasu (1952) and by Foster (1960). The original method is unsatisfactory in that the magnitude of recorded responses is small, and preparation of the chain is laborious. The subsequent modifications have overcome the first objection, but they have not eliminated the tedious preparation of a tracheal chain. The guinea-pig spirally cut tracheal strip, described here, is quickly and simply prepared, and is suitable for the investigation of spasmogens and their inhibitors.

Male guinea-pigs, 350 to 500 g, were used. The excised trachea was placed on gauze soaked with Krebs-Henseleit solution and cleaned of extraneous tissue. It was then cut, one end to the other, in spiral fashion such that 2 or 3 segments of cartilage separated each turn of the spiral. The entire strip can be used, or it can be cut in half thus providing two preparations from one donor. Each strip was suspended in a tissue bath containing Krebs-Henseleit solution at 38° aerated with 95% oxygen and 5% carbon dioxide. The strip was attached to a Grass FT.03 transducer, and contractions were recorded with a Grass polygraph. The tracheal strip contracted against an imposed tension of 5 g; less tension resulted in inconsistent results. Strips were left in the bath for 1 hr before starting an experiment; during this time the bathing medium was changed 3 to 4 times. Contractions to each spasmogen were elicited at 15 min intervals.

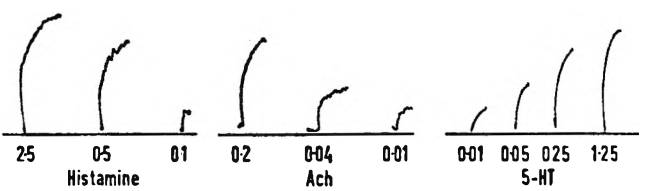

FIG. 1. Contractions of guinea pig spirally cut tracheal strips to: histamine, acetylcholine (Ach), 5-hydroxytryptamine (5-HT). Numbers refer to bath concentrations of spasmogens in μg/ml.

Fig. 1 shows responses of tracheal strips to histamine, acetylcholine, and 5-hydroxytryptamine. The strips were more sensitive to acetylcholine than to histamine. This agrees with Carlyle's (1963) finding with the guinea-pig tracheal chain, and with Jamieson's (1962) results with the isolated intact trachea. It is not in accord with Akcasu's (1952) finding that the tracheal chain is equally sensitive to histamine and acetylcholine. The sensitivity of the spirally cut tracheal strip to 5-hydroxytryptamine was approximately the same

as that to acetylcholine; Jamieson (1962) found the intact trachea less sensitive to 5-hydroxytryptamine than to acetylcholine.

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March 29, 1965

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An experiment in programmed learning

SIR,—The principle of programmed instruction, by which a student is given an item of information and his understanding of it is then checked by means of a question before passing on to the next item, is not new and was in fact the method employed by Socrates. However, in recent years interest in this form of teaching, with or without the use of machines, has increased but mainly at secondary school level. The Report of the Committee on University Teaching Methods (Hale, 1964) reviews the state of programmed learning in the universities to date and concludes that the method should be applicable at university level but that its usefulness 'will require to be tested by trial and error'. It would appear that at the present time very little use is made of programmed material for undergraduate teaching in this country, the main impetus coming from the U.S.A. Even there, usage is still at an early stage in medical and similar courses (Allender, 1964) and although a few excellent programmes have been published in book form (Wolf & Crowder, 1964; Nice, O'Connell & Sykes, 1964), these are expensive and still leave very few programmes of instruction available that are directly applicable to pharmaceutical subjects (Gerraughty, 1964). The aim was to produce and use a short programme and compare this method of teaching with the traditional undergraduate lecture.

A programme was produced consisting of 60 "frames" covering 38 quarto-sized pages concerned with the mechanisms of urine formation. The method of programming was that attributed to Crowder—fairly large units of information with questions at the end of each frame which allowed for the correction of wrong responses. The material was presented in scrambled form in such a manner that answers to the question at the end of any particular frame were not in immediate juxtaposition. The experiment was made with first year undergraduate pharmacy students. The programme was given instead of lectures to 44 of the 88 students chosen alphabetically and they were asked to complete it alone, but with free reference to textbooks. The remaining group of 44 students was given two normal 1 hr lectures, care being taken to cover during the course of the lecture every item of information included in the programme. After one week had elapsed both groups were given a 30 point objective test of a fairly searching nature designed to check both factual knowledge and understanding. An analysis of the results of a previous and more extensive examination had shown that the mean marks of the two groups were statistically indistinguishable. In addition those students who had read the programme were

asked questions designed to determine their attitude to this type of learning. The results are shown in Table 1.

TABLE 1. A COMPARISON BETWEEN A GROUP OF STUDENTS STUDYING A PROGRAMMED TEXT AND A SIMILAR GROUP RECEIVING LECTURES

	Programme group	Lecture group
Students tested after programme	42	
Students tested after lectures..		40
Mark from previous objective test (79 possible); mean ± s.e.	46·7 ± 1·1	46·5 ± 1·1
Mark from objective test on urine formation (30 possible); mean ± s.e.	*12·7 ± 0·6	11·1 ± 0·6
Hours spent on studying programme or in lectures; mean ± s.e. ..	2·4 ± 0·2	2·0
Additional hours declared spent in private study; mean ± s.e. ..	0·5 ± 0·1	1·2 ± 0·2

* Significantly different (P = 0·99).

Whilst obviously no far reaching conclusions can be drawn from a single trial such as this, it is evident that the students who read the programme were at no disadvantage compared to the lectured group and that the total time spent on the topic by the two groups was about the same. In fact the slight improvement shown by the programme-group is statistically significant compared with the control group receiving lectures. Of rather more interest was the attitude of the students to the programme method. 66% said they would like more programmes but often qualified this by saying that they should be in addition to and not instead of lectures. However, only 39% preferred the method to lectures and 32% said they enjoyed it less than lectures. Obviously the effort of writing a programme vastly exceeds that of preparing the equivalent lectures—Hale (1964) states 100 hours of preparation result in 1 hr of student study time—and if these results are typical the rewards would not seem to be comparatively large. The most common complaint from the students was that at the end of their period of study they were left without a set of lecture notes in a suitable form for subsequent revision.

However, in view of current criticisms of the lecture (Robbins, 1963; Hale, 1964) as the main method of conveying information and the current students demand for more individualistic modes of instruction, it is felt that more experiments with programmes covering a range of undergraduate subjects would be well worthwhile.

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Anti-anaphylactic effect of vitamin C in the guinea-pig

SIR,—Hoffman (1942) reported the anti-anaphylactic effect of vitamin C in mice. Naranjo (1952) and Herxheimer (1955) found that vitamin C has no protective effect against histamine shock in the guinea-pig at the specified dose levels.

The effect of vitamin C in histamine shock and anaphylaxis is here reported.

Guinea-pigs, 375–425 g at the beginning of the experiment, received 50 mg vitamin C per day in the drinking water for 21 days. Each week the animals were subjected to histamine or antigen aerosol using the technique of Herxheimer (1952), and animals found suitable in this way were chosen for the test. The histamine aerosol, 0·25 mg base/ml, was sprayed into a glass box. The animal was allowed to remain in the box until the first sign of coughing or choking occurred, and the preconvulsion time was recorded in seconds. The intracardial injection of the vitamin was not used, because the severe stress to the animals might have interfered with the test. The preconvulsion times (sec) for the following conditions were: control (injected with saline), 130 (s.d. 9·84); 200 mg vitamin C i.p., 20 min before anaphylaxis, 293 (s.d. 26·16); 200 mg vitamin C i.m., 2 hr before anaphylaxis, 223 (s.d. 18·63). Each figure is an average of 5 animals.

A group of 5 animals was tested for histamine resistance as above and then put on a scorbutic diet for 21 days and tested again. The preconvulsion times (sec) were: normal, 118 (s.d. 9·08); 8–12 day scurvy, 77 (s.d. 6·1); 18–21 day scurvy, 297 (s.d. 11·5).

Anaphylactic shock in vivo. The guinea-pigs were sensitised to commercial egg albumen by the intraperitoneal injection of 100 mg as a 5% w/v solution in normal saline. Three weeks later the animals were exposed to an aerosol of 50 mg of the antigen 1% w/v. The end-point was fixed at 10 min after which the animal was taken out of the box. When 200 mg vitamin C was administered intraperitoneally 20 min before being shocked, 7 out of 16 survived. In the control guinea-pigs only 2 out of 16 survived. When the dose of the antigen which caused shock was given intracardially, all the animals died. The time before death, however, was delayed 3–4 times in animals given the vitamin, compared with the controls.

No protective effect of vitamin C was found with oral administration of the vitamin or after 6 hr from the injection. The finding of Naranjo and Herxheimer that low doses of the vitamin had no protective effect, was also confirmed.

It is concluded that vitamin C has an anti-anaphylactic effect in the guinea-pig with the doses and under the conditions specified. At the early stage of scurvy there is a significant drop in the resistance of the animal to histamine aerosol and a marked rise above normal at the late stage of scurvy.

I thank Dr. P. B. Marshall for his help and advice.

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Effect of a mixture of dexamphetamine and amylobarbitone on critical flicker fusion frequency

SIR,—A proprietary mixture of dexamphetamine sulphate 5 mg and amylobarbitone 32·4 mg (Drinamyl) is claimed by its manufacturers to produce a therapeutic effect "without the drowsiness that accompanies the use of barbiturates alone, and without the irritation or anxiety that may accompany the use of stimulants alone". The opposing action of these drugs has been studied in man (Dickens, Lader & Steinberg, 1965) using simple mental and motor tasks, but no reference could be found to an investigation of their effect on a purely sensory modality such as the critical flicker fusion frequency (c.f.f.f.).

In a double-blind procedure, six normal subjects were given amylobarbitone 100 mg, amylobarbitone 100 mg + dexamphetamine sulphate 15 mg, and a placebo in random order based on a latin square design. Ascending and descending thresholds of c.f.f.f. at 2 and 4 hr were compared with readings before administration. C.f.f.f. was determined by exposing the subjects to intermittent light at 20 and 50 c/sec for 1 min before measuring the c.f.f.f. threshold using a neon lamp driven by a square wave oscillator as the light source. This has been found to increase the sensitivity of the method, and to allow a study of the effect of drugs not only on mean c.f.f.f. but also on the recently recognised adaptation phenomenon (Alpern & Sugiyama, 1961; Turner, 1964).

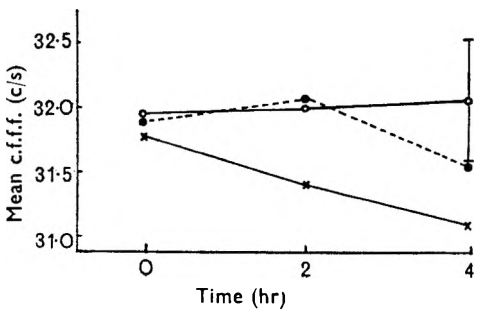

FIG. 1. Mean c.f.f.f. before and at 2 and 4 hr after administration of a placebo (● – ●), amylobarbitone 100 mg (× – ×) and a mixture of amylobarbitone 100 mg and dexamphetamine 15 mg (○ – ○) in 4 subjects. 95% confidence limits were all of the same order and are shown only for the mixture at 4 hr.

The results are shown in Fig. 1. There is a significant fall in mean c.f.f.f. over 4 hr after amylobarbitone 100 mg. The mixture of amylobarbitone and dexamphetamine, on the other hand, does not produce any significant change in c.f.f.f. during the period of the experiment, and it does not differ significantly from the placebo, although at 4 hr the difference between them is almost significant at the 5% level.

Neither amylobarbitone alone or in combination with dexamphetamine produced any change in the adapting effect of light at 20 and 50 c/sec.

This experiment demonstrates that dexamphetamine sulphate, 15 mg, abolishes the depressant action of amylobarbitone, 100 mg, on c.f.f.f. The difference in threshold at 4 hr between the mixture and the placebo, although not quite significant at the 5% level, suggests that at this time dexamphetamine may be having a dominant action. This may not only be due to the dose of dexamphetamine used in the combination but also to its rate of excretion which is

markedly dependent on changes in urinary pH (Beckett, Rowland & Turner, 1965). The duration of action of dexamphetamine in opposing depressant actions of amylobarbitone is probably closely related to urinary pH.

Roback, Krasno & Ivy (1952) found that dexamphetamine sulphate was effective in preventing the depression of c.f.f.f. produced by antihistamine drugs. It is equally effective in combination with amylobarbitone.

I am grateful to Mr. J. V. Smart for statistical help in this experiment which was carried out during the tenure of a Wellcome Senior Research Fellowship in Clinical Science. Smith Kline and French Laboratories Ltd. provided tablets of amylobarbitone, dexamphetamine and placebo.

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Use of time-response relationships in assessing pharmacological activity

SIR,—In many pharmacological experiments it is possible to obtain a continuous record of drug activity. Time-response curves provide an ideal basis for determining quantitative estimates of potency since they include a consideration of onset, peak and duration of action of the compounds under investigation. However, the use of time-response curves to obtain such estimates is seldom made. We would like to describe a quick and easy means of processing data for estimating the potency of compounds from integration of time-response curves.

There are two types of curve: one in which an effect is elicited in a single group of animals and its intensity assessed at several different times later, and an alternative in which different groups of animals are each examined once but at a variety of times, a relationship being built up between drug effect and time. For the purpose of this letter the method is applied to the assessment of anti-inflammatory activity of compounds in the guinea-pig ultraviolet erythema test described by Winder, Wax, Burr, Been & Rosiere (1958) but could be equally applied to many other pharmacological test procedures.

Male albino guinea-pigs of 250–400 g had an area 3 inches square on one dorsal flank depilated. Next day the guinea-pigs, in groups of 5, had test compounds administered orally, either dissolved or suspended in 5% w/v gum acacia, in a volume of 5 ml/kg. Control animals received gum acacia only. Two hr after drug administration, the flank of each animal was exposed to ultraviolet light for 30 sec. The head of the lamp was covered with a mask in which 3 holes of 6 mm diameter had been cut. The average intensity of the three resulting erythematous circles was estimated 1, 2 and 3 hr later using an arbitrary scoring system (slight erythema, 1; moderate erythema, 2; severe erythema, 3). For control animals and with each dose of test compound the responses from the group of 5-guinea-pigs were summed (group inflammatory score) and plotted against time. The relationship between group inflammatory score and time for a typical group of control animals is illustrated in Fig. 1A.

The area under the graph (integral) represents the continual level of inflammation over the 3 hr period of the test and is given by the formula overleaf, obtained from Fig. 1B.

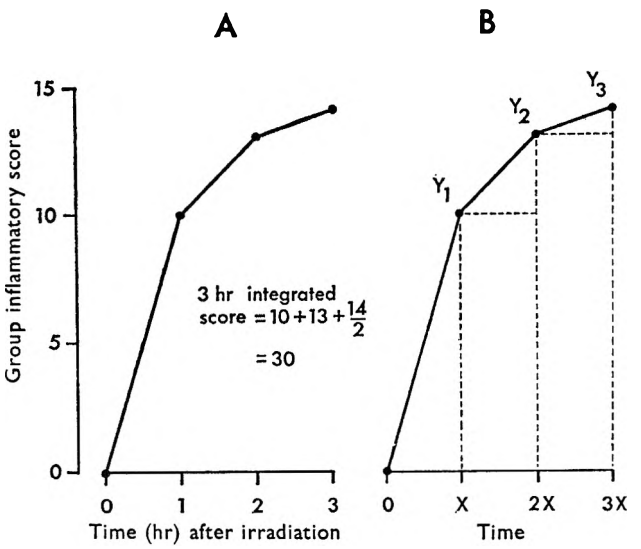

FIG. 1. Development of erythema in the guinea-pig. A, control animals. B, identical graph to show derivation of formulae for calculating area under the graph.

$$\text{Area} = \frac{XY_1}{2} + XY_1 + \frac{X(Y_2 - Y_1)}{2} + XY_2 + \frac{X(Y_3 - Y_2)}{2}$$

$$= XY_1 + XY_2 + \frac{XY_3}{2} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

TABLE 1. ACTIVITY OF ANTI-INFLAMMATORY DRUGS AGAINST ULTRAVIOLET-INDUCED ERYTHEMA IN THE GUINEA-PIG

Drug	Oral dose mg/kg	Group inflammatory scores at 1 hr	at 2 hr	at 3 hr	3 hr integrated score*	ED50 mg/kg
Phenylbutazone ..	0	12	14	14	33	10·8
	8	5	9	11	19·5	
	15	4	5	8	13	
	30	0	4	6	7	
Oxyphenbutazone	0	11	14	14	32	15·6
	8	6	9	13	21·5	
	15	6	6	10	17	
	30	2	5	7	10·5	
Aspirin	0	10	12	14	29	139
	50	6	10	13	22·5	
	100	5	8	12	19	
	200	2	4	9	10·5	
Sodium salicylate ..	0	11	14	15	32·5	244
	100	7	12	14	26	
	200	7	9	12	22	
	400	2	4	5	8·5	
Aminopyrine ..	0	9	12	15	28·5	105
	50	7	11	13	24·5	
	100	4	7	11	16·5	
	200	0	2	5	4·5	
Phenazone ..	0	10	13	14	30	265
	50	9	13	12	28	
	100	8	10	11	23·5	
	200	6	7	11	18·5	

* See Formula (2)

where Y_1, Y_2 and Y_3 are the successive group inflammatory scores at X time intervals. In the anti-inflammatory test described here, X = 1 (hr) and the formula simplifies to

$$\text{Area} = Y_1 + Y_2 + \frac{Y_3}{2} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

For each dose of test compound the integral is calculated and expressed as a percentage of that of control animals. Using probit-logarithmic graph paper this percentage plotted against the dose gives a linear relation from which the dose which reduces the integral of the control response by half (ED50) can be determined by inspection.

Table 1 summarises the results obtained with six known anti-inflammatory drugs. These results are in good agreement with the known therapeutic value of these anti-inflammatory drugs.

Formula (1) is applicable to an experiment in which three observations are made at constant intervals, but the formula can be easily adapted to an experiment in which n observations are made at X time intervals. The integration of the resulting time-response curves can be calculated from the general expression,

$$\text{Area} = XY_1 + XY_2 + XY_3 + \quad .. \quad .. \quad XY_{n-1} + \frac{X}{2} Y_n \quad .. \quad (3)$$

If the time interval between observations increases geometrically, the log interval is constant and is used for X in formula (3) in the calculation of the response integrals. In fact the general formula (3) applies to all time response relationships commencing at the origin irrespective of slope. It is concluded that the analysis has wide application in pharmacological test systems.

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Effects of ascorbic acid on the catecholamine content of guinea-pig myocardium

SIR,—When studying the effect of inhibitors of catechol-*O*-methyltransferase such as pyrogallol, and monoamine oxidase inhibitors such as nialamide or iproniazid, on the concentration of adrenaline or noradrenaline in auricles and ventricles of the guinea-pig heart, we injected ascorbic acid before the pyrogallol to prevent the latter causing a possible methaemoglobinaemia even at low doses (10 mg/kg, i.p.).

Adrenaline and noradrenaline were measured by the method of Bertler, Carlsson & Rosengren (1958). In this way we found that 10 min after an intraperitoneal injection of 500 mg/kg of ascorbic acid there was a significant decrease of adrenaline in both auricles and ventricles in unanaesthetised animals, there being no appreciable modification of the noradrenaline (Table 1).

This significant decrease of adrenaline in auricles and ventricles effected by ascorbic acid was also seen in guinea-pigs anaesthetised with urethane (1·2 g/kg, i.p.), when there was also a decrease of noradrenaline in auricles. Urethane alone

TABLE 1. CONTENT OF ADRENALINE AND NORADRENALINE IN HEART

Drugs	Auricles ± s.e.		Ventricles ± s.e.		Total heart ± s.e.	
	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
Control (8)* ..	0·566 ±0·074	4·237 ±0·241	0·474 ±0·039	2·170 ±0·156	0·520 ±0·070	3·203 ±0·363
Ascorbic acid (6)	0·209 ±0·037 P<0·001 (*d*)	4·112 ±0·664	0·101 ±0·046 P<0·001 (*d*)	2·378 ±0·293	0·155 ±0·025 P<0·001 (*i*)	3·245 ±0·465
Pyrogallol (12) ..	0·391 ±0·091	4·059 ±0·353	0·308 ±0·055	1·849 ±0·116	0·349 ±0·063	2·954 ±0·198
Ascorbic acid + pyrogallol (6) ..	0·667 ±0·063	3·549 ±0·152	0·458 ±0·020	2·093 ±0·274	0·522 ±0·036	2·820 ±0·199
Pyrogallol ..	P<0·01 (*i*)	P<0·02 (*d*)	P<0·001 (*i*)		P<0·01 (*i*)	
Ascorbic acid ..	P<0·01 (*i*)		P<0·001 (*i*)		P<0·001 (*i*)	P<0·001 (*d*)
Urethane (6) ..	0·396 ±0·080	4·548 ±0·325	0·422 ±0·127	1·641 ±0·361	0·409 ±0·103	3·094 ±0·091
Urethane + ascorbic acid (6) ..	0·205 ±0·027 P<0·001 (*d*)	3·110 ±0·283 P<0·02 (*d*)	0·210 ±0·061 P<0·01 (*d*)	1·913 ±0·178	0·207 ±0·031 P<0·001 (*d*)	2·511 ±0·213
Urethane + pyrogallol (6) ..	0·467 ±0·043	3·348 ±0·295	0·388 ±0·024 P<0·02 (*i*)	1·493 ±0·169	0·427 ±0·023 P<0·02 (*i*)	2·420 ±0·124
Pyrogallol ..						P<0·01 (*d*)
Urethane ..		P<0·02 (*d*)				P<0·01 (*d*)
Urethane + ascorbic acid + pyrogallol (6) ..	1·316 ±0·191 P<0·01 (*i*)	3·135 ±0·344	0·450 ±0·040 P<0·01 (*i*)	2·895 ±0·414	0·883 ±0·086 P<0·001 (*i*)	3·040 ±0·521

* Number of animals in parentheses.
(*i*) Increase.
(*d*) Decrease.

did not modify the myocardial catecholamine concentration. Pyrogallol alone, 20 min after intraperitoneal injection, also did not modify the catecholamine concentration of guinea-pig heart, but in animals treated with ascorbic acid it produced a significant increase of adrenaline in auricles and a highly significant increase of adrenaline in ventricles. These results occur both in unanaesthetised animals and in others anaesthetised with urethane.

According to McLean & Cohen (1963), ascorbic acid liberates adrenaline from the adrenal medulla granules *in vitro*, and this is antagonised by chelating agents. If this effect also occurs *in vivo*, and this has yet to be shown, the ascorbic acid-induced depletion of adrenaline in auricles and ventricles might be explained, as also might the increase of adrenaline in auricles and ventricles when both ascorbic acid and pyrogallol are given, as a consequence of an increase in extracellular catecholamines by inhibition of catechol-*O*-methyltransferase.

At the doses used, it is also possible that ascorbic acid could prevent the *in vivo* oxidation of pyrogallol, which would prolong its effects compared with the conditions when pyrogallol is given alone.

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March 23, 1965

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Research Papers

Letters to the Editor

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