RESEARCH PAPERS

THE INTERACTION OF CHELATING AGENTS WITH BACTERIA

PART II. CATION BINDING AND THE ANTIBACTERIAL EFFECTS OF 8-HYDROXYQUINOLINE (OXINE)*

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Received March 7, 1960

The binding of iron, cobalt and manganese ions from their aqueous solutions by viable and heat-killed suspensions of *Staph. aureus* is investigated. Competitive uptake of any two of these metal ions by viable and heat-killed organisms was also studied. The cation binding properties of viable and nonviable suspensions of *Staph. aureus* were compared and discussed. Data for the uptake of metal ions are also discussed in relation to the bactericidal effects of oxine solutions containing an equimolar concentration of iron and similar solutions containing additional iron, or cobalt or manganese.

WE have postulated previously[2] that the bactericidal action of solutions containing oxine and iron might be the consequence of the metal ion forming a bridge to link the oxine molecule to an important site in the cytoplasmic membrane of the bacteria. Since Rubbo, Albert and Gibson[3] have demonstrated the unique action of cobalt in reversing the bactericidal action of iron-oxine solutions against *Staphylococcus aureus* suspensions, the uptake of cobalt was studied under conditions similar to those used previously with iron. Manganese was also investigated as an example of a metal ion devoid of antibacterial activity in the presence of oxine and apparently incapable of reversing the toxic effects of iron-oxine solutions against *Staph. aureus* suspensions. Binding studies of the separate cations by *Staph. aureus* suspensions using solutions containing various combinations of cobalt and manganese, together with evaluation of the bactericidal action of oxine solutions containing one or more of these metal ions, were undertaken and similar uptake studies have been carried out using heat-killed bacterial suspensions.

Thus it was hoped that some pattern would emerge to contribute towards a greater understanding of the mechanism of antibacterial action of oxine.

METHODS

Reagents. Analar grade chemicals were used unless otherwise stated. 8-Hydroxyquinoline (oxine) was as described previously[2]. Solutions of ferrous ammonium sulphate ($FeSO_4(NH_4)_2SO_4.6H_2O$), cobalt sulphate ($CoSO_4.7H_2O$) and manganese sulphate ($MnSO_4.6H_2O$) in water were

* A summary of part of the work described in this paper was presented at the British Pharmaceutical Conference, September 1959[1].

prepared immediately before use. If oxine was added to any solution of a metallic salt, the mixture was stored at least 24 hours before use.

Precautions taken to minimise contamination with metal ions. Distilled water (subsequently referred to as water) was obtained from a Baracop still (Baird and Tatlock Ltd.). Glassware was treated with acid and alkali before use following the method of Waring and Werkman[4].

Spectrophotometer. A Hilger H 700 spectrophotometer was used in conjunction with matched fused silica cuvettes.

Preparation of bacterial suspensions. The test bacteria (*Staph. aureus*) were harvested from surface cultures with water; the resulting suspension was centrifuged at 8,500 *g* for 10 minutes and the cells resuspended and again washed with water. The final bacterial suspension was standardised nephelometrically to contain 10^{10} organisms/ml.

Preparation of suspensions of heat-killed bacteria. Washed suspensions of *Staph. aureus* in water were heated in flowing steam at 98–100° for 15 minutes. After centrifuging and washing once, the bacteria were resuspended in water and diluted to the original volume.

General method for the uptake experiments. Solutions containing either iron, cobalt or manganese, or oxine or a mixture of two or more of these substances (total volume 45 ml.), were introduced into glass centrifuge tubes which were immersed in a water bath at 25 $\pm$ 1°. After allowing sufficient time for temperature equilibration, 5 ml. portions of the standardised bacterial suspensions were added to each of the solutions to give a final concentration of 10^9 organisms/ml. One hour later, unless otherwise stated, the bacteria were removed by centrifuging and the supernatant solutions analysed quantitatively for the components of the initial solution.

Determination of unchanged oxine in solutions after contact with bacteria. Oxine was determined by measuring the optical density of the solution at 252 mμ after adding an equal volume of 0·2 N hydrochloric acid. This figure was corrected for the presence of cell exudate by measuring the optical density at 252 mμ of a portion of the solution after chloroform extraction to remove oxine and oxine-metal chelates (cf. reference 2 for procedure and reference 5 for precision).

Colorimetric determination of iron. The method was as described previously[2] except that 5 ml. instead of 2 ml. portions of the *o*-phenanthroline reagent were used. The optical densities of the solutions containing less than $0{\cdot}17 \times 10^{-4}$ M iron were measured in 4 cm. cuvettes; 1 cm. cuvettes were used for more concentrated solutions up to a maximum of $0{\cdot}8 \times 10^{-4}$ M iron. Neither cobalt nor manganese, at molar ratios to iron of 10:1 and 50:1 respectively, interfered with colour formation.

Colorimetric determination of cobalt. Cobalt was determined by the formation of a coloured complex with α-nitroso-β-naphthol-3,6-disulphonic acid (Nitroso R. salt): to a suitable volume of solution was added 5 ml. of 2 per cent v/v phosphoric acid (H_3PO_4), 5 ml. of 0·5 per cent. w/v solution of Nitroso R salt in water and 2·5 ml. of 50 per cent w/v solution of sodium acetate ($CH_3COONa.3H_2O$). The mixture was heated on a boiling water bath and 1 ml. of concentrated hydrochloric

acid added after 6 minutes; heating was continued for a further 4 minutes. The solution was cooled and diluted to 100 ml. with water. The optical density of the solution was measued at 530 mμ using 1 cm. cuvettes for solutions containing between $0{\cdot}13 \times 10^{-4}$ M and $0{\cdot}5 \times 10^{-4}$ M cobalt and 4 cm. cuvettes for more dilute solutions. A straight line relation was obtained by plotting the optical density at 530 mμ of the coloured

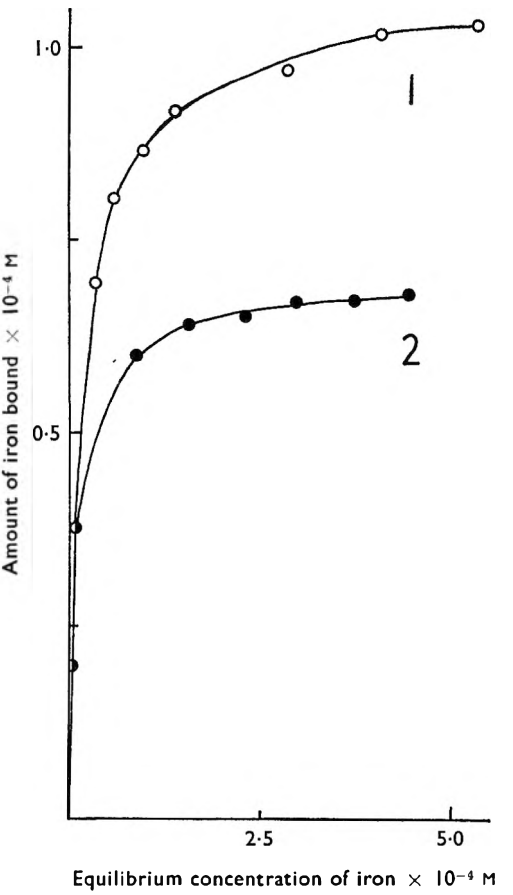

FIG. 1. The uptake of iron by viable (curve 1) and heat-killed (curve 2) suspensions of *Staph. aureus*.

solutions against the cobalt concentration. The colour was stable for at least 3 hours. Neither iron, up to molar ratio of iron to cobalt of 50:1, nor oxine nor an arbitrary concentration of cell exudate (cf. reference 5) interfered with the above method. The effect of widely varying proportions of manganese on the formation of the cobalt-nitroso R complex, using solutions containing between $6{\cdot}2 \times 10^{-6}$ M and $3{\cdot}0 \times 10^{-5}$ M cobalt, were investigated. The deviation from the cobalt calibration curve ($0{\cdot}062$–$0{\cdot}45 \times 10^{-4}$ M) was almost independent of the amount of

manganese ($0{\cdot}20$–$2{\cdot}5 \times 10^{-4}$ M) added to the solutions and of the relative molar proportions of the two metal ions.

Colorimetric determination of manganese. To a suitable volume of solution was added 10 ml. of concentrated nitric acid and 10 ml. of water. The solution was boiled gently for 10 minutes, 0·5 g. potassium periodate (KIO_4) was added and heating was continued by steaming for 60 minutes. The cooled solution was diluted to 100 ml. The optical density of the permanganate solution thus obtained was measured at λ max 525 mμ; 4 cm. cuvettes were used for solutions containing less than $0{\cdot}5 \times 10^{-4}$ M manganese and 1 cm. for those containing between $0{\cdot}5 \times 10^{-4}$ and $3{\cdot}0 \times 10^{-4}$ M manganese. A straight line relation was observed

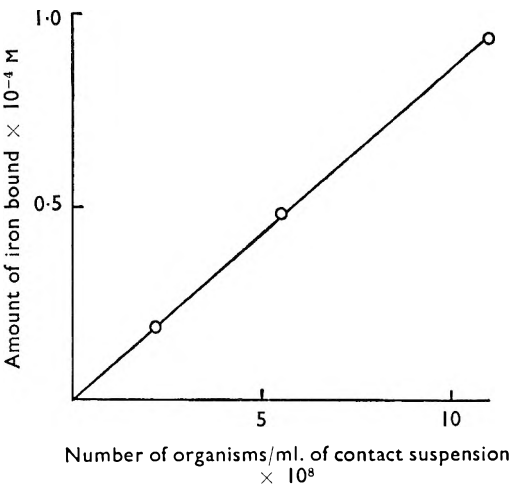

FIG. 2. The relation between the amount of iron bound and the number of organisms in the contact suspension.

on plotting the optical density at 525 mμ against the manganese concentration. Neither iron nor cobalt up to a molar ratio of iron or cobalt to manganese of 50:1, nor oxine nor an arbitrary concentration of cell exudate affected this method.

Bactericidal evaluation. The method was similar to that for the uptake measurements except that sterile materials were used and aseptic conditions were employed. Solutions containing oxine and metal ions, etc., were filtered through sintered glass filters (5/3) and the concentrations checked before use. Suspensions of *Staph. aureus* were prepared in sterile water and standardised to contain $11{\cdot}2 \times 10^8$ organisms/ml. 5 drops (0·09 ml.) of this suspension were added to 10 ml. volumes of the solutions under test (final bacterial concentration of 10^7 organisms/ml.) and 5 drop portions of this mixture were immediately transferred to dry sterile test tubes. The reactions were quenched at timed intervals by the addition of 5 ml. of sterile lemco-peptone broth and then incubated for 24 hours at 37° before examination for the presence of visible growth.

The experiments were replicated (usually 10-fold). Solution temperatures were maintained at 25 ± 1° before incubation. An oxine solution (1×10^{-5} M) containing an equivalent concentration of iron was included in each set of experiments to act as a daily standard. The percentage deviation of the extinction time observed for the bacteria in the standard oxine iron solution (44 ± 10 minutes) from the average was calculated and the results for the other solutions adjusted to take into account the day to day variations in the results.

RESULTS

The Uptake of Iron by Viable Staph. aureus *Suspensions*

The results obtained for solutions initially containing up to $6{\cdot}5 \times 10^{-4}$ M iron are represented by curve 1 of Figure 1. Curve 2 of the same diagram shows the results for a heat-killed suspension of the bacteria.

TABLE I

A COMPARISON OF THE UPTAKE OF COBALT AND MANGANESE BY VIABLE AND HEAT-KILLED *Staph. aureus* CELLS

	Initial concentration $\times 10^{-4}$ M	Uptake by	
		Viable organisms $\times 10^{-4}$ M	Heat-killed organisms $\times 10^{-4}$ M
Co^{++}	1·16	0·51	0·44
	1·86	0·54	0·52
	2·79	0·59	0·56
	4·66	0·65	0·62
Mn^{++}	2·55	0·85	0·77
	6·39	1·07	1·01
	7·66	1·11	0·99

Thus the maximum amount of iron bound is much reduced by heat-killing the organisms.

With an initial concentration of $5{\cdot}1 \times 10^{-4}$ M iron, an amount sufficient to allow the maximum uptake by the bacteria, the extent of iron binding was independent of the contact time between 20 and 60 minutes. Sorption was at least 90 per cent complete within 2 minutes.

Iron binding is proportional to the number of bacteria present, at least over a limited range, if the initial concentration is sufficient to attain maximum uptake (Fig. 2).

The Uptake of Cobalt by Staph. aureus *Suspensions*

The results for solutions containing up to $7{\cdot}0 \times 10^{-4}$ M cobalt initially are represented by curve 2 of the Mass Law plot, Figure 7. The graph relating the amount of cobalt bound by the bacteria to the equilibrium concentration of cobalt (see reference 1) showed that the maximum uptake of cobalt was much less than the maximum uptake of either iron or manganese.

The speed of cobalt binding by *Staph. aureus* suspensions was similar to that noted for iron with solutions initially containing $1{\cdot}77 \times 10^{-4}$ M cobalt.

The amount of cobalt bound was reduced slightly by heat-killing the bacterial suspension, as shown by the data in Table I. These results were obtained from a single suspension of the bacteria, a portion of which was heated; precautions were taken to minimise the loss of bacteria during the latter treatment.

The Uptake of Manganese by Staph. aureus *Suspensions*

A plot relating the amount of manganese bound to the equilibrium concentration of manganese is virtually superimposable on the curve

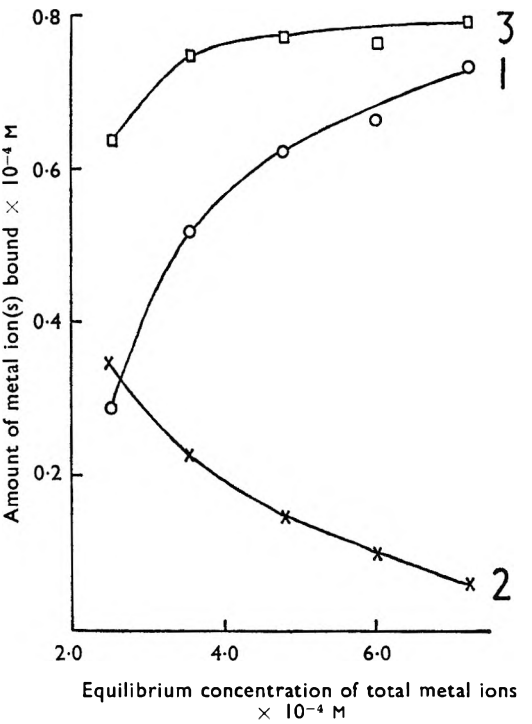

FIG. 3. Competitive binding of iron and cobalt by viable suspensions of *Staph. aureus*. The initial cobalt concentration was constant at $1{\cdot}8 \times 10^{-4}$ M.

Curve 1, the amount of iron bound.
2, the amount of cobalt bound.
3, the total amount of iron plus cobalt bound.

for iron (i.e. curve 1 of Fig. 1). A Mass Law plot of the data for manganese is included in Figure 7 (curve 1).

The speed of uptake of manganese from solution by *Staph. aureus* was similar to that noted for iron. The initial solutions contained $6{\cdot}7 \times 10^{-4}$ M manganese.

The relative amounts of manganese bound by viable and heat-killed suspensions of *Staph. aureus* are shown in Table I. These results, obtained from a single original bacterial suspension, indicate a slight reduction in manganese binding capacity in the heated suspensions.

The relative maximum amounts of iron, cobalt and manganese bound by a *Staph. aureus* suspension containing 10^9 organisms/ml. are $1{\cdot}07 \times 10^{-4}$ M, $0{\cdot}67 \times 10^{-4}$ M and $1{\cdot}06 \times 10^{-4}$ M respectively. These results were obtained using the same initial bacterial suspension; the total number of bacteria present in the contact suspensions was determined microscopically. These precautions were necessary as there were small day to day numerical variations in the bacterial suspensions standardised by the nephelometric method.

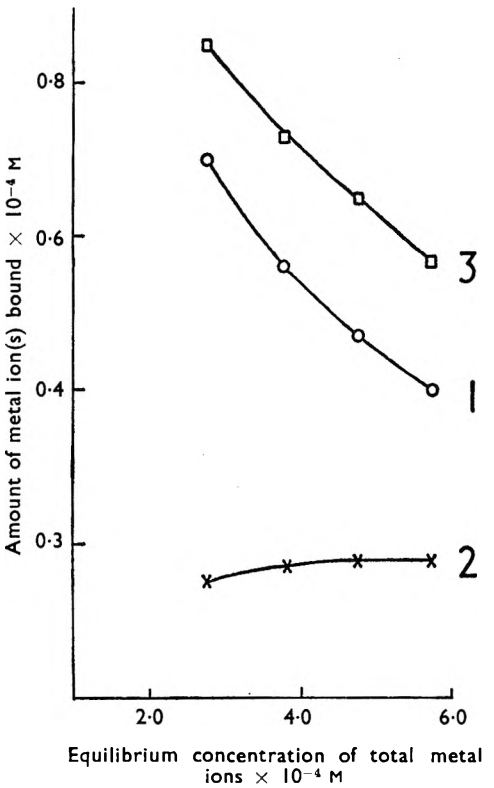

FIG. 4. Competitive binding of iron and cobalt by viable suspensions of *Staph. aureus*. The initial iron concentration was constant at $2{\cdot}7 \times 10^{-4}$ M.

Curve 1, the amount of iron bound.
2, the amount of cobalt bound.
3, the total amount of iron plus cobalt bound.

Competitive Binding of Metal Ions by Living Staph. aureus *Cells*

Iron and cobalt. Figure 3 shows the results obtained for the uptake of iron (curve 1) and cobalt (curve 2) from solutions initially containing a constant concentration of cobalt and varying proportions of iron. The maximum total amount of metal ions bound (curve 3) exceeded the maximum uptake of cobalt alone but was less than the corresponding value for iron alone.

The results for the uptake of iron (curve 1) and cobalt (curve 2) from solutions initially containing a constant concentration of iron and varying amounts of cobalt are presented in Figure 4. Curve 3 of the same diagram shows the total amount of the metal ions bound.

Iron and manganese. Figure 8 shows the plot of the amount of iron (curve 1), and of manganese (curve 2) and of total metal ions (curve 3) bound by a *Staph. aureus* suspension plotted against the equilibrium concentration of the total metal ions when the initial solution contained a constant concentration of manganese and varying proportions of iron. A similar graph was obtained when the iron content of the initial solutions

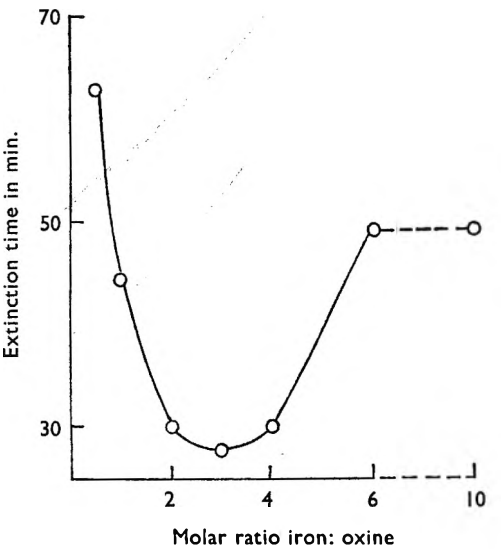

FIG. 5. Extinction times of *Staph. aureus* in solutions containing oxine (1×10^{-5}M) and varying proportions of iron.

was constant and the proportion of manganese was varied. The maximum total amount of bound iron and manganese corresponded to the maximum uptake of either of the individual metal ions in both cases.

Cobalt and manganese. The results for this system were exactly analogous with those for the iron and cobalt competition studies described above.

Competitive Binding of Metal Ions by Heat-killed Staph. aureus *Cells*

Iron and cobalt. The results for iron and cobalt binding by heat-killed organisms from solutions containing both ions are similar to those described above for iron and manganese binding by viable *Staph. aureus* suspensions; the maximum concentration of metal ions bound, however, is lower. Figure 9 shows the results for iron and cobalt binding by *Staph. aureus* suspensions using solutions initially containing varying concentrations of cobalt and a constant concentration level of iron.

Iron and manganese. Figure 10 shows the results obtained when the manganese content of the initial solution was kept constant and the iron

concentration was varied. The result was similar when this position was reversed. The maximum combined total uptake of iron and manganese is about 1 per cent less than the corresponding value for manganese on heat-killed organisms.

Cobalt and manganese. The results were similar to those obtained for the competition studies with iron and manganese with heat-killed suspensions of *Staph. aureus*.

Bactericidal evaluation. The results for the bactericidal evaluation of oxine solutions containing varying proportions of iron are summarised in Figure 5.

The effect of adding cobalt to the oxine-iron solution is shown in Figure 6; addition of a one or two molar equivalents of cobalt (cobalt: oxine: iron of 1 : 1 : 1 or 2 : 1 : 1) caused a slight potentiation of bactericidal

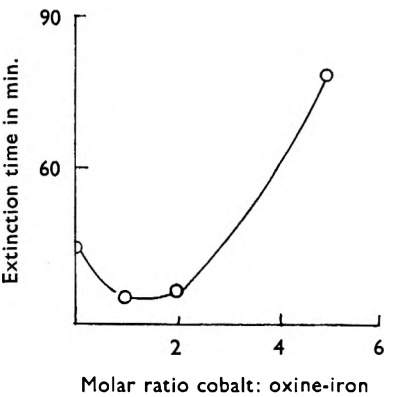

FIG. 6. Effect of cobalt on the extinction time of *Staph. aureus* in a solution containing oxine and iron (both at 1×10^{-5} M).

activity against *Staph. aureus*, whereas, a five molar equivalent of cobalt caused a reduction in activity.

No reduction in activity was observed in solutions containing oxine and iron and manganese at ratios of 1 : 1 : 1, 1 : 1 : 2 and 1 : 1 : 5.

Solutions containing oxine and either cobalt or manganese (up to a five molar equivalent) were non-toxic to *Staph. aureus* under similar conditions.

DISCUSSION

Cation Binding by Viable Staph. aureus *Cells*

Iron. When the initial iron concentration was sufficient to achieve maximum uptake by the bacteria, binding was almost complete within 2 minutes of contact. This is in contrast to the results reported previously[2] using lower concentrations of iron in the contact suspensions in which the metal ion uptake remained incomplete after 60 minutes contact with bacteria.

Presentation of the results for iron binding by a *Staph. aureus* suspension as a Mass Law plot (Rothstein and Hayes[6]) is made in curve 1 of Figure 7. Since two intersecting straight lines are obtained, it may be concluded

that two types of binding site are involved. This supports the previous postulation of anionic and chelating sites for iron binding by this organism[2]. The results of the binding studies using solutions containing iron and cobalt also support this postulate.

Cobalt. The maximum amount of cobalt bound by *Staph. aureus* suspensions is about 70 per cent less than the corresponding amount of iron (see Table I) although the speed of uptake is similar. The Mass

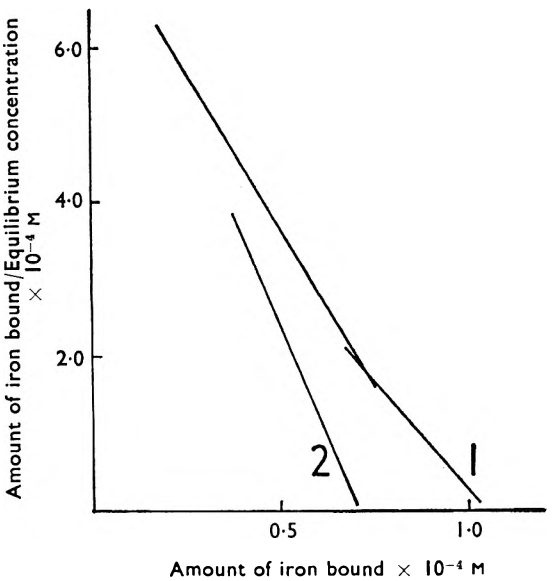

FIG. 7. Mass Law plots for the uptake of manganese or iron (curve 1) and cobalt (curve 2) by viable suspensions of *Staph. aureus*.

Law plot of the uptake data, a straight line (curve 2 of Fig. 7), indicates that there is only one type of binding site for cobalt on the bacterial surface.

Manganese. The marked similarity between the results for iron binding by viable *Staph. aureus* suspensions and those for manganese suggested the existence of a common binding site for these ions on the bacterial surface.

TABLE II

SUMMARY OF THE NUMBER OF TYPES OF CATION BINDING SITES ON *Staph. aureus*

Metal ion	Viable organisms	Heat-killed organisms
Fe^{++}	2	1
Co^{++}	1	1
Mn^{++}	2	2

The number of metal ions bound per bacterium at maximum uptake was estimated; the values obtained were $6{\cdot}5 \times 10^7$ atoms per bacterium for iron and manganese and 4×10^7 atoms per bacterium for cobalt.

Cation Binding by Heat-killed Staph. aureus *Cells*

The maximum amount of iron bound by *Staph. aureus* suspensions was much reduced by heat-killing the organisms (see Fig. 1); the seeming *slight* reductions in cobalt and manganese binding by heat-killed suspensions compared with viable suspensions may be disregarded since some losses of bacteria must inevitably occur during the preparation of the former. Since the light scattering properties of bacterial suspensions

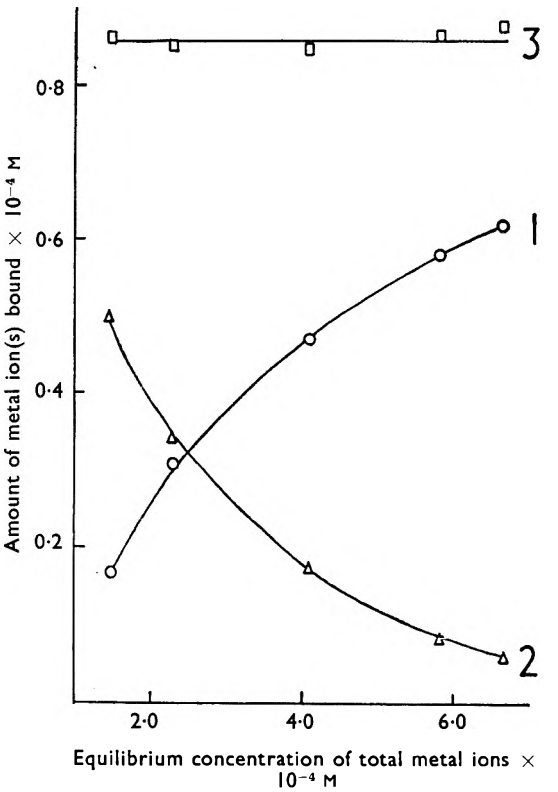

FIG. 8. Competitive binding of iron and manganese by viable suspensions of *Staph. aureus*. The initial manganese concentration was constant at $1{\cdot}67 \times 10^{-4}$ M.

Curve 1, the amount of iron bound.
2, the amount of manganese bound.
3, the total amount of iron plus manganese bound.

change during heat treatment, corrections would be applicable only if viable and total counts were made on the suspensions before and after heating.

Thus with *Staph. aureus* suspensions the maximum amount of iron bound by viable organisms is reduced by heat-killing to a level similar to that observed for cobalt-binding by the same bacteria (viable or heat-killed). The extent of manganese binding was hardly affected by similar treatment.

Mass Law plots of the uptake data for the three metal ions by heat-killed *Staph. aureus* suspensions indicated the number of binding sites summarised in Table II. The results of the competition studies using heat-killed bacteria support these conclusions.

Competitive Binding of Metal Ions by Living Staph. aureus *Cells*

Iron and cobalt with constant initial cobalt concentration. The data presented in Figure 3 show that the amount of cobalt bound decreases as the amount of iron bound increases. The greatest amount of both is about 0·75–0·80 $\times$ 10^{-4} M, whereas, the corresponding figure for

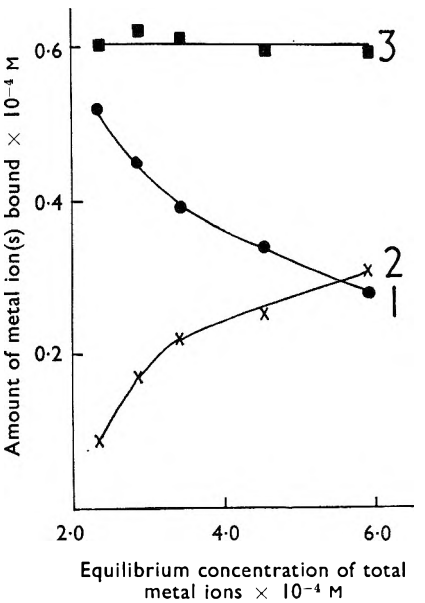

FIG. 9. Competitive binding of iron and cobalt by heat-killed suspensions of *Staph. aureus*. The initial iron concentration was constant at 2·40 $\times$ 10^{-4} M.

Curve 1, the amount of iron bound.
2, the amount of cobalt bound.
3, the total amount of iron plus cobalt bound.

iron is 0·95–1·00 $\times$ 10^{-4} M and for cobalt 0·65–0·70 $\times$ 10^{-4} M. Assuming that both ions are bound at a common site and that a second site is available for iron binding, then the maximum metal ion uptake of the two should correspond to the maximum uptake of iron. But this was not found. Therefore, cobalt ions, either bound or free, apparently interfere with normal binding at the second iron site.

Iron and cobalt with constant initial iron concentration. The above suggestion is further supported by the results (Figure 4) which show that the amount of iron bound by *Staph. aureus* suspensions is reduced as the initial cobalt concentration is increased, whereas, the amount of cobalt bound does not alter significantly. This indicates that the *free*

cobalt ions appear to exert a great effect on the extent of iron binding by this organism.

Iron and manganese. Since similar plots are obtained for iron and manganese binding by *Staph. aureus* suspensions from solutions containing both metal ions, irrespective of which metal is kept at a constant initial concentration (see Fig. 8), it may be deduced that these metal ions are bound at similar sites. Further, the combined amount of iron and manganese bound is equivalent to the maximum uptake of either of these ions alone. By interpolation, the ratio of iron to manganese in the initial solution at the point of intersection of curves 1 and 2 in Figure 8 is 1 : 1; a similar value was also obtained from the data for iron and manganese binding by *Staph. aureus* when the iron content of the initial solution was kept constant.

Cobalt and manganese. The remarks above concerning iron and cobalt also apply to the results obtained for the cobalt and manganese studies with viable organisms.

Competitive Binding of Metal Ions by Heat-killed Staph. aureus *Cells*

Iron and cobalt. Iron and cobalt ions appear to be interchangeably bound at a single site on the bacterial surface (Fig. 9); the maximum total uptake of the two ions corresponds approximately to the maximum uptake of either of the individual ions by heat-killed suspensions of this organism. Iron is bound to a greater extent than cobalt in the proportion of 1 : 0·67. Although changes must occur in the bacterial surface during the heat treatment it may be assumed that these metal ions share a common binding site on the heated bacteria as well as on the viable organisms. Thus the major effect of heating appears to be a modification of the second iron binding site, i.e. the chelating site.

Iron and manganese. Interchangeability of binding of these two ions by heat-killed suspensions of *Staph. aureus* is evident from the results presented in Figure 10. The relative proportions of iron and manganese present in the initial solution from which equivalent concentrations of the two ions are bound by the bacteria indicate that iron is bound preferentially to manganese at a ratio of 1 : 0·84. Since iron is bound preferentially and at only one type of site by heat-killed suspensions of *Staph. aureus*, whereas, manganese is bound at two types of sites (Table II), the slight reduction in the maximum uptake level for the combined metal ions compared with the corresponding figure for manganese binding was not unexpected.

Comparison of Cation Binding by Viable and Heat-killed Staph. aureus *Cells*

Table II shows the number of binding sites for iron, cobalt and manganese available on the surface of the bacteria. The loss of the second iron binding site after heat-killing the bacteria was not unexpected as some molecular rearrangement of the bacterial surface might be anticipated (cf. reference 7). However, the lack of change in the manganese binding sites contradicts this suggestion especially since iron

and manganese ions were interchangeably bound by the viable bacteria. These results might be explained by steric effects or the oxidation state of the metal ions. A change in the avidities of the binding groups on the bacterial surface could be the result of a rearrangement of intermolecular bonding brought about by heat. Changes in the physical orientation of groups at the surface could obviously alter the nature

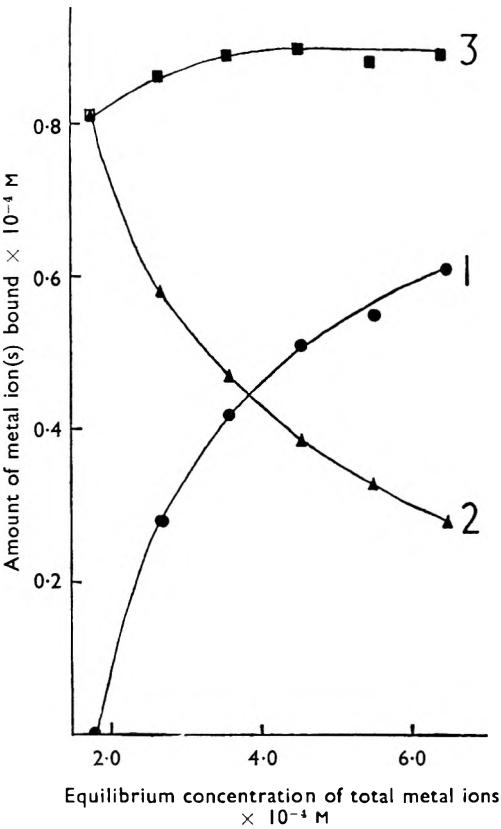

FIG. 10. Competitive binding of iron and manganese by heat-killed suspensions of *Staph. aureus*. The initial manganese concentration was constant at 2·57 × 10^{-4} M.

Curve 1, the amount of iron bound.
2, the amount of manganese bound.
3, the total amount of iron plus manganese bound.

and extent of ions or molecules subsequently bound thereto. Alternatively, if a change in the oxidation state of some of the metal ions occurs at the bacterial surface and is a pre-requisite for their binding, heat-inactivation of the enzyme system responsible for the change could prevent binding of the metal ions presented in an oxidation state unacceptable to the organism.

The Effect on Bactericidal Properties

Some trends may be noted from the results: (i) that the optimum bactericidal activity against *Staph. aureus* was exhibited by solutions containing iron and oxine at a molar ratio of 1 : 3,

(ii) that addition of an equivalent molar ratio of cobalt to the iron-oxine solution causes a slight potentiation in bactericidal activity, whereas, a five mole excess of cobalt reduced the toxicity of the 1 : 1 iron-oxine solution to *Staph. aureus* and gave a solution which was also less toxic than a solution containing oxine and a five mole equivalent of iron,

(iii) that addition of manganese to the iron-oxine solution only slightly affected the bactericidal activity against *Staph. aureus*.

Although the object of this work has been incompletely realised, there may be some correlation between the bactericidal results for oxine-metal ion solutions and the competition studies using solutions containing mixtures of two metal ions. For example, if the toxic effect of iron in the presence of oxine is a consequence of iron binding at one particular site, then a five molar equivalent of cobalt may displace sufficient iron atoms to reduce the toxicity. Also, with the interchangeably bound iron and manganese ions, the latter may be unable to displace the former from the sites where it exerts its toxic effect so that the bactericidal activity is hardly affected by relatively small proportions of manganese.

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THE SYNTHESIS OF SOME POTENTIAL ANTIMETABOLITES OF PHENYLALANINE

PART II. THE SYNTHESIS OF SOME ββ-DIALKYL-α-AMINOPROPIONIC ACIDS

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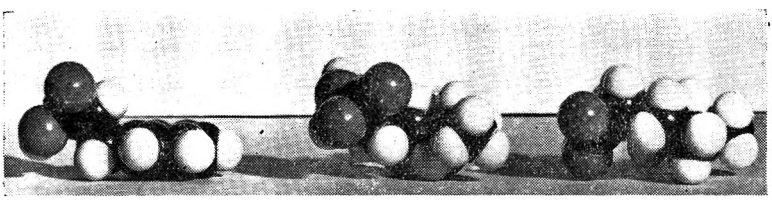
Received March 1, 1960

Seven ββ-dialkyl-α-aminopropionic acids, including five new compounds, have been synthesised as potential antiviral and antibacterial agents. None showed any significant activity against the Newcastle Disease virus *in vitro*. Several methods of preparation of the compounds have been investigated, and theories are advanced to explain the failure of some of these methods.

PHENYLALANINE is an essential metabolite for the Influenza A and Newcastle Disease viruses[1] (Dickinson, personal communication), and in Part I[2] we reported the synthesis of some γγ-dialkyl-α-aminobutyric acids as possible but unsuccessful antagonists of phenylalanine in the Newcastle Disease virus- Chick chorio-allantoic membrane system.

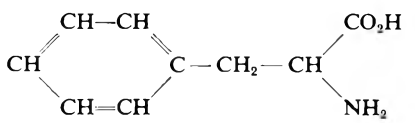
Continuing this work, in which we are trying to formulate and synthesise possible theoretical antagonists of phenylalanine, we have now prepared a similar series of ββ-dialkyl-α-aminopropionic acids.



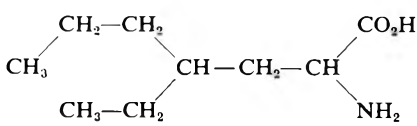
1 2 3

FIG. 1. Catalin models of β-phenylalanine (1), α-amino-γ-ethyl-γ-propylbutyric acid (2) and α-amino-β-ethyl-β-propylpropionic acid (3).

Catalin models of these compounds (Fig. 1) indicate that their bulk more nearly approaches that of phenylalanine (I) than does that of the γγ-dialkyl-α-aminobutyric acids from which they differ only in the length of the main carbon chain, which is reduced from three to two carbon atoms.

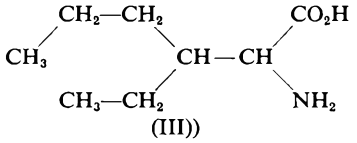


(I)



(II)

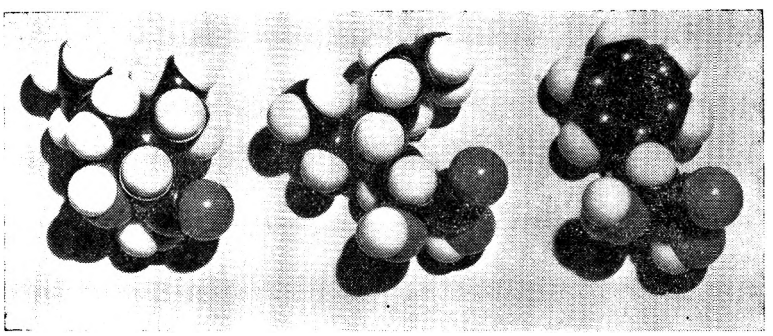
α-Amino-γ-ethyl-γ-propylbutyric acid



(III))

α-Amino-β-ethyl-β-propylpropionic acid

However, the absence of the methylene group between the two carbon atoms carrying the polar and non-polar residues results in an appreciable change in the spatial configuration of the molecule compared to that of phenylalanine (Fig. 2*a* and *b*).



1 2 3

FIG. 2A. Catalin models of α-amino-β-ethyl-β-propylpropionic acid (1), α-amino-γ-ethyl-γ-propylbutyric acid (2) and β-phenylalanine (3).

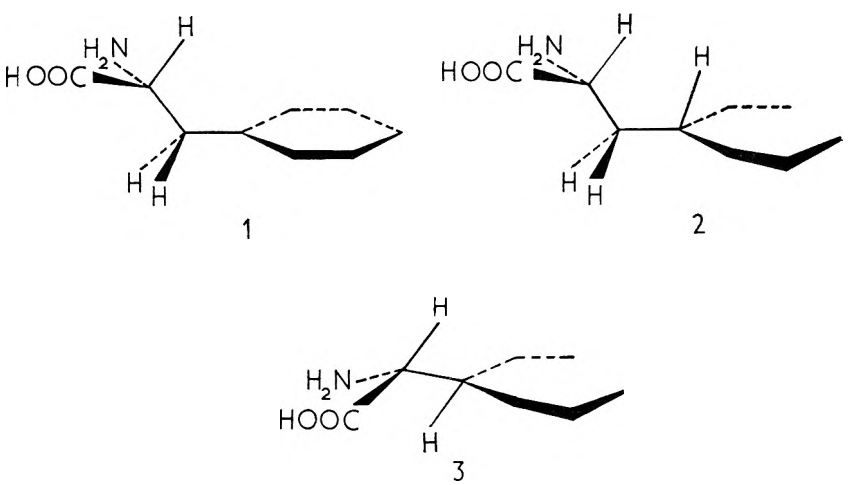


FIG. 2B. Steric conformations of phenylalanine (1), α-amino-γ-ethyl-γ-propylbutyric acid (2), and α-amino-β-ethyl-β-propylpropionic acid (3).

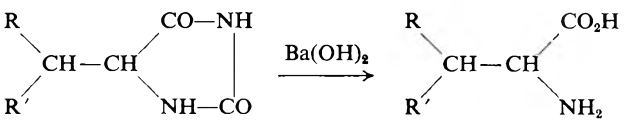
The model of phenylalanine shows that the amino-acid moiety is above the plane of the aromatic ring and a similar structure can be attained by the γγ-dialkyl-α-aminobutyric acids where the amino-acid moiety is above the plane of the alkyl groups, which themselves may approach a

ring structure. But in the case of the $\beta\beta$-dialkyl-α-aminopropionic acids, the absence of the methylene group ensures that the carbon atom carrying the two polar groups is in a very similar plane to that of the non-polar residue, and the polar groups themselves are directed slightly below this plane (Fig. 2*a* and *b*). Therefore, from a theoretical chemotherapeutic viewpoint, it is possible that this series of amino-acids may be utilised in the virus protein synthesis instead of phenylalanine, because of the similarity in the bulk and the chemical nature of the compounds. Thus antagonism may be effected by virtue of the difference in the spatial relationship of the polar and non-polar residues, which may result in the formation of a foreign protein and therefore interfere with virus protein synthesis.

Preparation of the Compounds

The parent of the $\beta\beta$-dialkyl-α-aminopropionic acids is the naturally occurring amino-acid valine, for which many syntheses are available. Several of these appear to be readily applicable to the synthesis of the higher homologues and consequently no difficulty was envisaged in the preparation of this series of compounds. However, we found that the higher $\beta\beta$-dialkyl-α-aminopropionic acids cannot be synthesised by the following procedures: (i) the alkylation of diethyl acetamidomalonate; (ii) the α-carbon nitrosation of alkylmalonates and alkylacetoacetates; (iii) hydrolysis and subsequent reduction of the α-hydroxyamino-nitriles; (iv) Schmidt's reaction with alkylmalonic esters; (v) Curtius' degradation of alkylcyanacetic esters.

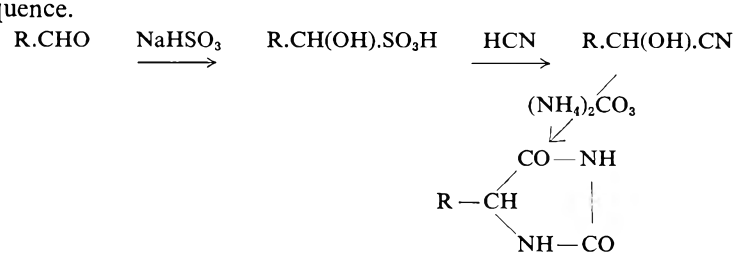
The compounds were eventually prepared by the hydrolysis of the 5-(dialkylmethyl)hydantoins (IV).



(IV)

The various methods available for the synthesis of the 5-alkylhydantoins have been reviewed by Ware[3] and of these, two are seemingly easily applicable to the preparation of the 5-(dialkylmethyl)hydantoins, namely the Bucherer-Bergs' synthesis from carbonyl compounds and the condensation of hydantoin with carbonyl compounds and subsequent reactions.

The Bucherer-Bergs' synthesis is outlined by the following reaction sequence.



The appropriate 2,2-dialkylacetaldehydes are the desired starting materials for the preparation of the 5-(dialkylmethyl)hydantoins, but of those required, only 2-ethylhexanal is commercially available. Others, as indicated in the relevant literature, have often been obtained as by-products in other investigations, for instance, Sutter and Wijkmann[4] isolated 2-ethylvaleraldehyde from the degradation of glauconic acid, while investigating the chemistry of moulds and Bunner and Farmer[5] obtained 2-isopropyl-2-methylacetaldehyde from the ozonolysis of olefin condensation products. Two apparently general methods for the synthesis of 2,2-dialkylacetaldehydes appear in the literature, but in each, the starting materials are difficult to obtain. Sommelet and his colleagues[6–8] prepared 2-methylvaleraldehyde and 2-propylvaleraldehyde by heating the appropriate 2,2-dialkylethylene glycols or their 1-ethyl ethers and Sou[9] reported a new aldehyde synthesis involving the distillation of the hydrobromides of 2,2-dialkyl-2-dialkylaminoethanols, from which he obtained some of the corresponding 2,2-dialkylacetaldehydes.

Of the standard methods for the preparation of aldehydes, only the Rosenmund reduction appears to have been applied to these compounds (excluding isobutyraldehyde), and although Cason and Reinhart[10] reported a 71 per cent yield of 2-methyldodecanal from the corresponding acid chloride, Discherl and Nahm[11] obtained only 7 per cent of 2-ethylisovaleraldehyde by this procedure. We failed to obtain either of the corresponding aldehydes from 2-butylhexanoylchloride and 2-ethylvalerylchloride by the Rosenmund method.

Furthermore, we were also unable to prepare the compounds by the Stephen procedure, the reaction of the appropriate Grignard compounds with ethyl orthoformate or by the reduction of acid halide derivatives with aluminium lithium hydride according to Weygand and his colleagues[12–14].

As a result of these unsuccessful attempts to synthesis the necessary 2,2-dialkylacetaldehydes, only 5-(3-heptyl)hydantoin was prepared by the Bucherer-Bergs' method, being obtained in 65 per cent yield from 2-ethylhexanal. The hydantoin was successfully hydrolysed with barium hydroxide octahydrate at 160° for 30 minutes to give α-amino-β-butyl-β-ethylpropionic acid (2-amino-3-ethylheptanoic acid) in 85 per cent yield.

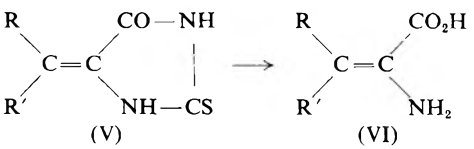
Consequently recourse had to be made to the second method available for the preparation of the required hydantoins.

Hydantoin has a reactive methylene group in the 5 position and therefore, might be expected to condense with carbonyl compounds which are reactive to such groups. Most of the past work in this field has been carried out with aldehydes, Wheeler and Hoffmann[15] first showing that hydantoin would condense with benzaldehyde in the presence of glacial acetic acid and sodium acetate to yield the 5-arylidene derivative, and soon afterwards, Wheeler and Brauchtlecht[16] and Johnson and Scott[17] showed that 2-thiohydantoin would condense similarly and often more readily than the oxygen analogue. Boyd and Robinson[18] introduced the use of a basic condensing agent instead of the acid medium used previously, finding that 1-acetyl-2-thiohydantoin would condense with

benzaldehyde in the presence of piperidine and pyridine or diethylamine and pyridine. Morpholine has also been used in similar condensations[19]. It has been shown that the nature of the secondary base is sometimes important, for Boyd and Robinson[18] obtained only 14 per cent of anisylidenehydantoin in the presence of piperidine–pyridine, whereas a diethylamine–pyridine medium gave 94 per cent of the same compound. 1-acetyl-2-thiohydantoin condensed with anisaldehyde equally well in either medium. The indifference of 2-thiohydantoin to the nature of the basic condensing agent was also demonstrated by Beer and his colleagues[20] who observed that piperidine–pyridine and morpholine–pyridine gave equally good yields of 5-isopropylidene-2-thiohydantoin from 1-acetyl-2-thiohydantoin and acetone. Our work on 5-(2-pentylidene)-2-thiohydantoin has shown that it is immaterial whether piperidine or diethylamine is used with pyridine for the condensation.

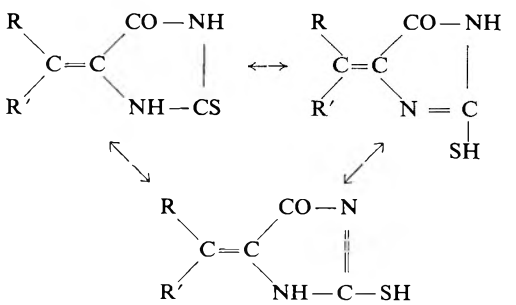
A few reports of the condensation of aliphatic ketones with 2-thiohydantoin have been made[20,21] and Yale[22] successfully condensed seven ketones with 2-thiohydantoin in good yields, although unlike Beer[20] and Dupré[21] he could not condense the ketones with 1-acetyl-2-thiohydantoin.

We have successfully condensed a further six aliphatic ketones with 2-thiohydantoin by refluxing the reactants for 1 hour in a mixture of piperidine and pyridine, followed by standing overnight. It was hoped that the hydrolysis of the condensates (V) would lead to the formation of the unsaturated amino-acids (VI).



(V) (VI)

Hydrolysis however, merely produced a cleavage at the double bond in an analagous manner to that described by Doyle, Holland and Naylor[23] who obtained thiohydantoic acid by the loss of the alkylidene group from 5-isopropylidene-2-thiohydantoin, on treatment with dilute alkali. In our case, glycine, identified by paper chromatography, was isolated.



Reduction to the saturated hydantoin before hydrolysis avoids this cleavage, and Ware[3] lists several methods for the reduction of the 5-alkylidenehydantoins to their corresponding saturated derivatives. As

the ease of reduction depends to some extent on the nature of the 5-alkyl group, it was felt, that in this instance, catalytic reduction would be the most suitable. However, the reduction of 5-(2-pentylidene)-2-thiohydantoin failed with both palladised charcoal and Raney nickel. This was attributed to the possibility of tautomerism in the molecule leading to thiol group formation, which probably leads to poisoning of the catalyst.

This problem was easily overcome by the conversion of the thiohydantoins to their oxygen analogues. Of the many reagents which have been used for this desulphurisation, aqueous chloracetic acid, introduced by Johnson, Pfau and Hodge[24] seemed the most satisfactory as it does not interfere with the alkylidene double bond as do the oxidising agents hydrogen peroxide and bromine water, which have also been used for this purpose. Refluxing the 5-alkylidene-2-thiohydantoins with 50 per cent aqueous chloracetic acid gave excellent yields of the oxygen derivatives, 5-(2-hexylidene)-2-thiohydantoin in particular giving a quantitative yield of 5-(2-hexylidene)hydantoin.

The 5-alkylidenehydantoins were readily reduced at room temperature and normal pressure using Raney nickel prepared by the method of Tucker[25], hydrogen uptake being completed after about 12 hours. This compared favourably with the results of Beer[20], who hydrogenated 5-isopropylidenehydantoin at 50 atmospheres pressure at 60° and Doyle[23], whose hydrogenation of the same compound at atmospheric pressure took 40 hours.

The saturated hydantoins were hydrolysed to the corresponding amino-acids by autoclaving with aqueous barium hydroxide at 160° for 30 minutes.

Examination of the results indicates that the inability to obtain some of the amino-acid intermediates was due to steric factors.

In the case of the alkylation of acetamidodiethylmalonate, Snyder, Shekelton and Lewis[26] found that they could not obtain the alkylated derivative from either isopropyl or secondary butyl bromide. Atkinson and Scott[27] obtained valine by this route, by using a prolonged reacton period, albeit in low yield, but they were unable to synthesis isoleucine by this route. Construction of models indicates the likelihood of steric hindrance, which increases with the molecular weight of the substituting secondary alkyl group.

Similar factors operate during the Curtius degradation of the secondary alkyl substituted malonic esters. Russel[28] found, that while he could ammonate monoethylmalonic ester to give 80 to 90 per cent of the amide, he could only obtain 3·7 per cent of diethylmalonamide and he attributed this to the steric nature of the diethylmalonic ester. The secondary alkyl malonic esters are very similar in structure to the dialkylmalonic esters and the reaction of the former with hydrazine is analogous to the reaction of the latter with ammonia.

Again, in the *C*-nitrosation of the alkylmalonic esters and alkylacetoacetates, the size of the substituting alkyl group appears to determine whether or no the reaction takes place. In the case of the compounds

with a primary alkyl group, nitrosation occurs readily, but although we successfully prepared the ethyl ester of 2-methyl-1-oximinobutyric acid by the method of Shivers and Hauser[29], which was then reduced to valine with palladised charcoal, the application of the method to the higher members of the series was completely unsuccessful.

In the case of the hydantoins, however, the precursors of the polar residues of the amino-acid are held in the rigid structure of the heterocyclic ring while the alkyl group is introduced. Similarly, in the preparation of α-amino-β-ethyl-β-propylpropionic acid by Gol'dfarb, Fabrichnyi

TABLE I

DIALKYLMETHANOLS, R.CH(OH).R′

R	R′	Yield per cent	B.p. found	B.p. lit.	n_D^{20}	Reference
CH_3	C_4H_9	72	139°	137–138°	1·4135	31
C_2H_5	C_3H_7	75	132–134°	134–136°	1·4140	32
C_4H_7	C_3H_7	76	151–152°	154°	1·4200	33
C_5H_{11}	C_4H_9	71	169–171°	71° 10mm.	1·4250	34
C_4H_9	C_4H_9	70	193–194°	194°	1·4290	35

and Shalovina[30], where the compound is synthesised by the desulphurisation of (1,5-dimethylthienyl-3)aminoacetic acid, the non-polar part of the molecule is held in the ring whilst the amino-acid moiety is added.

These results therefore suggest, that the higher homologues of valine can only be synthesised when the free movement of the polar and non-polar residues of the intermediates is reduced by clamping one end of the amino-acid precursor in a rigid ring structure.

TABLE II

ALIPHATIC KETONES, R.CO.R′

R	R′	Yield per cent	B.p. found	B.p. lit.	n_D^{20}	Reference
CH_3	C_4H_9	71	124–125°	127°	1·4005	36
C_2H_5	C_3H_7	64	123°	123°	1·4010	32
C_3H_7	C_3H_7	76	144°	144°	1·4080	37
C_3H_7	C_4H_9	75	166–168°	165–168°	1·4160	38
C_4H_9	C_4H_9	83	186–188°	187–188°	1·4198	35

Biological Results

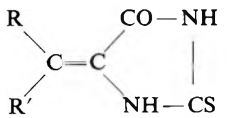
The 7 amino-acids were tested against Newcastle Disease virus in tissue culture using monolayers of chick embryo. None of the compounds showed any significant activity. BM 21 and 31 were inactive at a concentration of 1·7 mg./ml. BM 27, 38, 39 and 43 gave varying results at 1·7 mg./ml. but were all inactive at one fifth of this dose. BM 47 was toxic at 1·7 and 0·34 mg./ml. and inactive at 0·07 mg./ml.

EXPERIMENTAL

Typical syntheses are described. Details for the individual compounds are given in Tables I to VI.

TABLE III

5-ALKYLIDENE-2-THIOHYDANTOINS

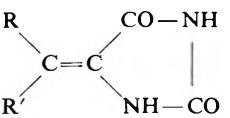


R	R′	Yield per cent	M.p.	Analysis Found C	Found H	Found N	Required C	Required H	Required N
CH_3	C_3H_7	87	157°*a*	52·3	6·8	15·4	52·2	6·5	15·2
CH_3	C_4H_9	72	130°*b*	54·0	7·3	14·1	54·5	7·1	14·1
C_2H_5	C_3H_7	50	148–149°	54·7	7·2	14·3	54·5	7·1	14·1
C_3H_7	C_3H_7	35	147–150°	57·0	7·9	13·3	56·6	7·6	13·2
C_3H_7	C_4H_9	28	118–119°	58·6	7·8	12·4	58·4	8·0	12·4
C_4H_9	C_4H_9	25	126°	59·9	8·4	12·2	60·0	8·3	11·7

All recrystallisations were carried out from 50 per cent aqueous ethanol.
[a]Yale[22], gives m.p. 152° from toluene. Recrystallisation of our sample from toluene gave m.p. 157°.
[b]Yale[22], gives m.p. 112–114° from toluene. Recrystallisation of our sample from toluene gave m.p. 130°.

TABLE IV

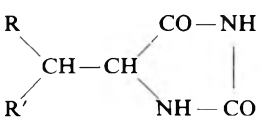
5-ALKYLIDENEHYDANTOINS



R	R′	Yield per cent	M.p.	Analysis Found C	Found H	Found N	Required C	Required H	Required N
CH_3	C_3H_7	84	170°	57·0	7·5	16·7	57·2	7·1	16·7
CH_3	C_4H_9	100	150°	59·0	7·8	15·6	59·3	7·7	15·4
C_2H_5	C_3H_7	78	155°	59·2	7·9	15·2	59·3	7·7	15·4
C_3H_7	C_3H_7	85	158°	61·6	8·2	14·5	61·2	8·2	14·3
C_3H_7	C_4H_9	94	134°	63·4	8·8	13·1	62·9	8·6	13·3
C_4H_9	C_4H_9	88	155°	64·5	8·7	12·5	64·3	8·9	12·5

TABLE V

5-ALKYLHYDANTOINS



R	R′	Yield per cent	M.p.	Method of preparation	Analysis Found C	Found H	Found N	Requierd C	Requierd H	Requierd N
CH_3	C_3H_7	90	119–120°	*b*	56·3	8·0	16·8	56·5	8·2	16·5
CH_3	C_4H_9	97	98–100°	*b*	59·1	8·5	15·3	58·7	8·7	15·2
C_2H_5	C_3H_7	94	170°	*b*	59·0	8·5	15·1	58·7	8·7	15·2
C_2H_5	C_4H_9	65	125–126°	*a*	61·1	9·4	14·0	60·6	9·1	14·2
C_3H_7	C_3H_7	94	160°	*b*	60·5	8·8	14·5	60·6	9·1	14·2
C_3H_7	C_4H_9	94	134°	*b*	62·3	9·5	13·3	62·3	9·4	13·2
C_4H_9	C_4H_9	75	99°	*b*	64·4	9·6	12·4	63·8	9·7	12·4

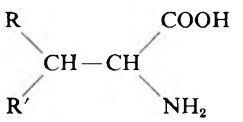
Method of Preparation. *a* from the aldehyde via the cyanhydrin.
b from the reduction of the 5-alkylidene.

Method A

5-(3-*Heptyl*)*hydantoin.* 2-Ethylhexanal (11·5 g.) was shaken with a solution of sodium metabisulphite (11 g.) in water (30 ml.). The resulting bisulphite compound was filtered off, sucked dry, and then treated with sodium cyanide (5 g.) in water (15 ml.) in three portions, cooling after each addition. The 3-butyl-3-ethyl-2-hydroxyacetonitrile which separated as an oil was removed and the aqueous residue extracted with benzene (20 ml.). The organic liquors were combined and extracted with 10 per cent aqueous sodium metabisulphite (25 ml.), and the benzene removed

TABLE VI

ββ DIALKYL-α-AMINOPROPIONIC ACIDS



Code No.	R	R′	Yield per cent	M.p.*	Analysis					
					Found			Required		
					C	H	N	C	H	N
BM 27	CH_3	C_3H_7	76	230–233°	57·5	10·5	10·1	57·9	10·5	9·7**
BM 31	CH_3	C_4H_9	91	235–239°	60·1	10·5	8·9	60·4	10·7	8·8
BM 38	C_2H_5	C_3H_7	76	248–249°	59·7	10·6	8·8	60·4	10·7	8·8***
BM 21	C_2H_5	C_4H_9	85	238–240°	62·8	10·7	7·9	62·4	11·0	8·1
BM 39	C_3H_7	C_3H_7	73	248–252°	61·9	10·8	8·1	62·4	11·0	8·1
BM 43	C_3H_7	C_4H_9	64	236–238°	64·2	11·5	7·6	64·2	11·3	7·5
BM 47	C_4H_9	C_4H_9	82	240–243°	65·1	11·4	6·7	65·7	11·4	6·9

* Melting points all occurred with decomposition
** Reported by Gagnon and others[39]
*** Reported by Gol'dfarb and others[80]

over a water bath. To the residual oil ammonium carbonate (40 g.) in 50 per cent aqueous ethanol (300 ml.) was added and the mixture heated at 55° for 3 hours, followed by heating to reflux for 5 minutes. The solvent was removed under reduced pressure, leaving a white solid which crystallised from 50 per cent aqueous ethanol to yield 5-(3-*heptyl*)-*hydantoin* (11·6 g., 65 per cent), m.p. 125–126°.

Method B

Hexan-2-ol. To magnesium (26 g.), in ether (300 ml.), was added butyl bromide (5 g.), and the mixture vigorously stirred until the formation of the Grignard complex commenced. The remainder of the butyl bromide (132 g.) was then added over 1½ hours and the mixture gently refluxed for 30 minutes. Acetaldehyde (44 g.) was added at a rate which just maintained reflux, and then the mixture was refluxed for 3 hours. The complex was decomposed by iced dilute hydrochloric acid, the ether layer separated and the aqueous residue extracted with ether (50 ml.). The ethereal liquors were combined and washed with 50-ml. portions of water, 10 per cent aqueous sodium carbonate and water. After drying over anhydrous sodium sulphate the ether was removed, and the residue fractionally distilled to yield hexan-2-ol (78 g., 72 per cent), b.p. 139°.

Butyl methyl ketone. Hexan-2-ol (76 g.) was added slowly to a mixture of sodium dichromate (180 g.) in concentrated sulphuric acid (150 g.)

and water (900 ml.), with vigorous stirring. After cooling, the upper layer was separated and the aqueous layer extracted twice with ether (50 ml.). The ethereal liquors were combined, dried over anhydrous sodium sulphate, the ether removed and the residue distilled to yield butyl methyl ketone (53·5 g., 71 per cent., b.p. 124–125°.

5-(2-*Hexylidene*)-2-*thiohydantoin*. 2-Thiohydantoin (10 g.), in a mixture of pyridine (30 ml.), and piperidine, (30 ml.) was refluxed with butyl methyl ketone (20 g.) for 1 hour. After standing overnight, the solvents and excess ketone were removed under reduced pressure, the residue dissolved in water (50 ml.), cooled in ice, and acidified with concentrated hydrochloric acid. The oil which separated solidified after standing overnight at 0°, and the resultant solid was filtered off and recrystallised from 50 per cent aqueous ethanol-charcoal to yield 5-(2-hexylidene)-2-thiohydantoin (12·2 g., 72 per cent), m.p. 130°.

5-(2-*Hexylidene*)*hydantoin*. 5-(2-Hexylidene)-2-thiohydantoin (10 g.) was refluxed with 50 per cent aqueous chloracetic acid (50 ml.) for 1 hour. After cooling, the white crystals were filtered off, and recrystallised from 50 per cent aqueous ethanol to yield 5-(2-*hexylidene*)*hydantoin* (10 g., 99 per cent), m.p. 150°.

5-(2-*Hexyl*)*hydantoin*. 5-(2-Hexylidene)hydantoin (8 g.) was hydrogenated in the presence of Raney nickel (8 g.) in ethanol (50 ml.) at atmospheric pressure and room temperature until the theoretical amount of hydrogen had been taken up (about 12 hours). The catalyst was filtered off, the solvent removed, and the resulting solid recrystallised from 50 per cent aqueous ethanol to yield 5-(2-*hexyl*)*hydantoin* (7·8 g., 97 per cent), m.p. 98°–100°.

α-*Amino*-β-*butyl*-β-*methylpropionic acid* (2-*amino*-3-*methylheptanoic acid*). 5-(2-Hexyl)hydantoin (5 g.) was autoclaved at 160° for 30 minutes with barium hydroxide octahydrate (12 g.) suspended in water (70 ml.). After filtration, carbon dioxide was passed into the mixture until precipitation of barium carbonate was complete. The solution was filtered, evaporated to dryness under reduced pressure, and the residue recrystallised from 50 per cent aqueous ethanol to yield α-*amino*-β-*butyl*-β-*methylpropionic acid* (3·4 g., 78 per cent., m.p. 243–248° with decomposition.

Acknowledgements. The authors are indebted to the Pharmaceutical Society of Great Britain for the award of the Wellcome Research Scholarship (to B. J. M.), and to Dr. L. Dickinson and Mr. P. Oxley and the Boots Pure Drug Co. for biological evaluation and chemical analyses. We should also like to thank Professor W. H. Linnell for encouragement.

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THE EFFECT OF CORTICOSTEROID ANALOGUES ON THE THYMUS GLAND OF THE IMMATURE RAT

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Received March 21, 1960

The thymolytic activity of hydrocortisone (cortisol) was increased approximately 9-fold by the introduction of a fluorine atom at either the 6α- or the 9α-position. The substitution of a methyl group in the α-position at carbons 2, 6 or 16, with one exception, enhanced the ability of the corticosteroid to involute the thymus gland 3- to 4-fold. The only exception was noted when a methyl group at carbon 2 failed to increase the potency of fludrocortisone (9α-fluorohydrocortisone acetate). The formation of a double bond at the 5,6-position of the steroid molecule consistently decreased the thymolytic activity. Although the addition of an hydroxyl group in the α-position at carbon 16 decreased the relative potency of the corticosteroids, the formation of the 16α, 17α-acetonide derivative markedly enhanced it.

The involution of the thymus gland of the immature rat which occurs as a result of the parenteral administration of an adrenal corticosteroid has been shown to be associated with the following molecular configuration: a ketone group at carbon 3, a double bond at the 4,5 position, an oxygen function at carbon 11 and an α-ketol side chain at carbon 17[1]. In addition, it was found that various alterations of the corticosteroid molecule, such as the addition of an α-hydroxyl at carbon 17, a double bond at the 1,2 position, or a fluorine at the 9α-position, enhanced the thymolytic activity[2].

The present communication is a continuation of earlier studies[2], and is concerned with the effect of substitution at carbons 1, 2, 6, 9 and 16 of the corticosteroid molecule on thymic involution.

Experimental

Immature male or female albino rats, 25 to 30 days of age, were weighed and distributed at random in groups of equal numbers. The average body weight in each group was equalised by appropriate exchange of animals between groups. These rats were originally of a Wistar strain which has been inbred in the animal colony of the Food and Drug Laboratory since 1928.

The thymus involution assay procedure employed in this study was modified slightly from that described earlier[1,2]. The corticosteroids were administered on the basis of the initial body weight, mg. of steroid per 100 g. of weight, and either two or three unknown compounds were assayed against the reference standard. Usually the relative potency of one of the "unknowns" had been determined previously, and this served as a check on the reproducibility of the assay procedure. A "2 × 2" design was used when three unknowns were tested, and a "3 × 3" design when two unknowns were assayed against the same reference compound.

The log dose interval for the "2 × 2" design was 0·3010, while that for the "3 × 3" design was 0·1761. The steroids were dissolved in maize oil and injected subcutaneously in the back, 3 times daily for a period of 2 days.

The calculations for estimating the potency ratios and their confidence limits were made either by the procedure outlined by Bliss[3] or that by Gaddum[4]. If an observation appeared to be an outlier, a rejection criterion based on the range[5] was applied to the assay data. Tests for significance and homogeneity of the individual slopes between assays were employed in all of the experiments. In addition to these tests, linearity

TABLE I

EFFECT OF INTRODUCING A FLUORINE ATOM AT THE 6- AND 9-POSITIONS ON THE RELATIVE POTENCY OF THE CORTICOSTEROIDS

Compound	Relative potency (equimolar basis)	Confidence limits (P = 0·95)
Hydrocortisone	1·0	
6α-Fluorohydrocortisone	8·9	8·3–9·5
6α, 9α-Difluoroprednisolone	101·1	94·0–108·9
Hydrocortisone acetate	1·0	
9α-Fluorohydrocortisone acetate	8·8	8·0–9·7
9α-Fluoroprednisolone acetate	17·1	16·9–17·3
2α-Methylhydrocortisone	1·0	
2α-Methyl-9α-fluorohydrocortisone acetate	2·1	2·0–2·2

of the individual log dose response lines was checked in the "3 × 3" assay design. A common slope was used to calculate each potency ratio, and the error term was estimated to include the variation of the treatment means about the log dose response lines. Since all of the responses were included in the estimation of the error term, a higher degree of precision was obtained than would have been possible if each compound had been assayed individually against the reference standard. The index of precision, obtained by dividing the standard deviation of the assay (s) by the common slope (b) was consistently less than 0·10. Usually two or more estimates of the potency ratio for each corticosteroid analogue were combined by the method of Bliss[3]. Homogeneity of the individual potency ratios was checked by the χ^2 test before the potency ratios were combined to provide the weighted mean value.

Results and Discussion

The data in Table I show the effect of the thymolytic activity of a fluorine atom at the 6α- or 9α-position of the corticosteroid molecule. The potency values relative to hydrocortisone (cortisol) suggest that a fluorine at carbon 6 has approximately the same action as one at carbon 9. The combination of a double bond at the 1,2-position and a fluorine at carbon 9 increased the thymolytic activity of hydrocortisone 17-fold. When a second fluorine was added at carbon 6 of 9α-fluoroprednisolone (6α, 9α-difluoroprednisolone) the potency was found to be approximately 100

times that of hydrocortisone. However, this augmentation in activity attributable to the addition of fluorine appears to be modified by changes in the molecular configuration at carbons 1 and 2. Relative potency values reported previously revealed that prednisolone is 4 to 5 times more active than hydrocortisone[2]. Therefore, the relative potency value of 17·1 shown in Table I for 9α-fluoroprednisolone indicates that the fluorine at carbon 9 brought about a 4-fold increase in the ability of prednisolone to produce thymic involution. Also, the potency of 2α-methylhydrocortisone was doubled only by the introduction of a fluorine at the 9α-position. In contrast, the addition of an α-fluorine at carbon 9 of hydrocortisone acetate, a steroid without changes at carbons 1 and 2, produced a 9-fold increase in the thymolytic activity.

TABLE II

EFFECT OF A DOUBLE BOND AT THE 5,6-POSITION ON THE THYMOLYTIC ACTIVITY OF THE CORTICOSTEROIDS

Compound	Relative potency (equimolar basis)	Confidence limits (P = 0·95)
Cortisone acetate	1·0	
6-Dehydrocortisone acetate	0·93	0·88–0·98
Hydrocortisone acetate	1·0	
6-Dehydrohydrocortisone acetate	0·79	0·75–0·84
6-Dehydroprednisolone acetate	1·9	1·8–2·0
6-Dehydroprednisone acetate	1·2	1·1–1·3
9α-Fluoroprednisolone acetate	17·1	16·9–17·3
9α-Fluoro-6-dehydroprednisolone acetate	4·4	4·1–4·8
9α-Fluoro-6-dehydrocortisone acetate	1·9	1·7–2·1
1-Chloro-6-dehydroprednisone acetate	0·0*	

* Activity absent at total dose level of 2·3 mg./100 g. of rat.

The relative potency values given in Table II reveal that a double bond between carbons 5 and 6 consistently reduced the ability of the corticosteroids to cause involution of the thymus gland. In addition, the data suggest that the lowered biological activity attributable to the 6-dehydro configuration was more pronounced in the corticosteroids with a fluorine at the 9α-position. According to the results obtained in Table II, substitution of a chlorine atom at carbon 1 further decreased the thymolytic action because 1-chloro-6-dehydroprednisone acetate did not possess any demonstrable activity when administered to the rats at a total dose of 2·3 mg./100 g. of weight. Under similar circumstances, hydrocortisone, in a total dose of 0·3 to 0·4 mg./100 g. of weight, would produce a significant decrease ($P \leqslant 0{\cdot}05$) in the relative thymus weight.

The results in Table III illustrate the effect of methyl group substitution on the relative potency of various corticosteroids. The introduction of a methyl group at the 2α-position of hydrocortisone, or at the 6α-position of either prednisolone or triamcinolone brought about a 3- to 4-fold increase in the thymolytic activity. Reference to both Tables I and III shows that the addition of a methyl group at the 16α- or 16β-position of 9α-fluoroprednisolone increased the potency relative to hydrocortisone from 17·1 to 72·7 and 51·7 respectively. According to these data the

introduction of a methyl group at the 16α-position enhanced the activity approximately 4-fold. On the other hand, a β-methyl group at carbon 16 produced a 3-fold increase in the relative potency. However, the results in Table III also reveal that the addition of an α-methyl group did not augment the thymolytic activity in every instance, because 2α-methyl-9α-fluorohydrocortisone acetate was actually less active than 9α-fluorohydrocortisone acetate.

TABLE III

INFLUENCE OF METHYL GROUP SUBSTITUTION ON THE RELATIVE POTENCY OF THE CORTICOSTEROIDS

Compound	Relative potency (equimolar basis)	Confidence limits (P = 0·95)
Hydrocortisone	1·0	
2α-Methylhydrocortisone	4·4	4·2–4·7
6α-Methylprednisolone	15·2	14·4–16·1
6α-Methyltriamcinolone	9·0	8·1–10·0
9α-Fluoro-16α-methylprednisolone	72·7	67·9–77·9
9α-Fluoro-16β-methylprednisolone	51·7	46·6–57·3
Prednisolone	1·0	
6α-Methylprednisolone	3·8	3·5–4·1
9α-Fluorohydrocortisone acetate	1·0	
2α-Methyl-9α-fluorohydrocortisone acetate	0·86	0·80–0·93

TABLE IV

EFFECT OF AN HYDROXYL GROUP AT THE 16α-POSITION, AND OF 16α, 17α-ACETONIDE FORMATION ON THE THYMOLYTIC ACTIVITY OF THE CORTICOSTEROIDS

Compound	Relative potency (equimolar basis)	Confidence limits (P = 0·95)
Hydrocortisone	1·0	
16α-Hydroxyhydrocortisone	0·13	0·10–0·17
16α-Hydroxyprednisolone	1·1	0·94–1·3
9α-Fluoro-16α-hydroxyprednisolone (triamcinolone)	3·8	3·6–4·0
9α-Fluoroprednisolone-16α-21-diacetate	3·6	3·1–4·2
6α-Methyl-9α-fluoro-16α-hydroxyprednisolone	9·0	8·1–10·0
6α-Methyl-9α-fluoroprednisone-16α-21-diacetate	4·7	4·1–5·4
Triamcinolone acetonide*	76·6	61·2–96·0
6α-Methyltriamcinolone acetonide	115·5	102·9–129·7
6α-Methyltriamcinolone-21-acetate-acetonide	97·1	86·5–108·9

* 9α-Fluoro-16α, 17α-isopropylidenedioxyprednisolone.

The data in Table IV demonstrate that an α-hydroxyl group at carbon 16 depressed the action of the corticosteroids on the rat thymus. This observation is based on the results obtained with the 16α-hydroxy derivatives of hydrocortisone, prednisolone, and 9α-fluoroprednisolone. Apparently acetylation of the hydroxyl groups at carbons 16 and 21 have no significant effect on the potency of the 16-hydroxycorticosteroid. In contrast to the depression of the ability to involute the thymus gland of the rat brought about by 16-hydroxylation of the corticosteroid, formation of the 16α, 17α-isopropylidenedioxy(16α, 17α-acetonide) derivative markedly enhanced the thymolytic action of these compounds. The

degree of augmentation achieved by acetonide formation appeared to be dependent on other groups present on the corticosteroid molecule. For example, triamcinolone acetonide is approximately 20 times more active than the free alcohol or diacetate, while the 6α-methyltriamcinolone acetonide derivative showed only a 10- to 12-fold increase in relative potency when compared with the parent compound. It is interesting to note that 6α-methyl-9α-fluoroprednisone 16α, 21-diacetate is only one-half as active as 6α-methyl-9α, 16α-hydroxyprednisolone (6-methyltriamcinolone). This is somewhat unexpected because previous work[2] has shown that the thymolytic activity of prednisone is approximately 70 to 75 per cent of that of prednisolone. Perhaps, the presence of the other groups on the molecule has modified the effect of the hydroxyl group at carbon 11[2].

During the course of this discussion, no attempt has been made to compare the results obtained by the thymus involution assay procedure with those used by other investigators for estimating glucocorticoid-like activity. In many instances either the route of administration or the injection medium has differed from that employed in the assays reported in this paper. Earlier work[1,2], has shown that the injection medium influenced the relative potency of some of the corticosteroid analogues as measured by the thymus involution assay. Ideally the various assays for glucocorticoid-like activity should be carried out simultaneously in test animals from the same group, and using the same route of administration and injection medium for all of the tests.

Acknowledgements. The author wishes to express his thanks to Dr. L. I. Pugsley for his kind interest in this investigation and to Mr. A. J. Bayne and Miss C. A. McLeod for their very valuable technical assistance. The steroids used in this study, were kindly supplied by Chas. Pfizer & Co., Inc., The Upjohn Co., Lederle Laboratories Division of the American Cyanamid Co., E. R. Squibb & Sons, Merck & Co., Inc., and The Schering Corporation.

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THE IMPORTANCE OF THE 19-METHYL AND THE C(20) KETONE GROUPS FOR THE THYMOLYTIC ACTIVITY OF THE ADRENOCORTICAL STEROIDS

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Received March 21, 1960

Evidence is presented to show that reduction of the ketone grouping at carbon 20 in the α-ketol side chain of the adrenocortical steroid to form either the α- or β-hydroxy derivative, produced a profound, if not complete, loss in the ability of the analogue to involute the thymus gland of the immature albino rat. The 19-nor-derivative of hydrocortisone (cortisol) was unable to produce a significant decrease in the thymus weight at the doses employed, suggesting that the 19-methyl is one of the groups which are essential for the thymolytic activity of the corticosteroids. A 19-norhydrocortisone derivative in which the 4-dehydro-3-keto grouping in ring A was replaced by a phenolic type of structure similar to that found in the oestrogenic steroids had no effect on the thymus gland of the rat.

Earlier work[1] has shown that adrenal corticosteroids which are capable of producing thymic involution in the rat possess the following molecular configuration: a double bond between carbons 4 and 5, a ketone group at the 3-position, an oxygen function at carbon 11, and an α-ketol side chain at carbon 17. This conclusion was based on the observation that steroid compounds which lacked one or more of these groupings did not induce a significant decrease in thymus weight of the test animals even when administered at relatively high doses.

In the present study, particular attention has been directed to the effect of the ketone group at carbon 20 in the α-ketol side chain and of the 19-methyl group on the thymolytic activity of hydrocortisone (cortisol) and some of its analogues.

Experimental

Intact 25 to 30-day old rats, derived from an inbred Wistar strain and raised in the animal colony of the Food and Drug Directorate, were used. The steroids were dissolved in maize oil and administered subcutaneously in the back three times daily for 2 days. On the third day the thymus glands were removed and weighed, and the thymus weight in mg./100 g. of final weight was recorded for each rat.

By using this procedure it was possible to obtain a significant involution of the thymus gland with a total dose of hydrocortisone ranging from 0·3 to 0·4 mg./100 g. weight.

Results

The data in Table I show that replacement of the ketone at carbon-20 of hydrocortisone by either an α-hydroxyl or a β-hydroxyl produced a marked decrease in the ability of the corticosteroid analogue to induce

thymic involution. In fact none of the 20-hydroxycorticosteroids produced a significant decrease in the relative thymus weight at dose levels 20 to 30 times greater than the minimum effective dose of hydrocortisone. Likewise the 19-nor compounds had no effect on the thymus glands of the

TABLE I

EFFECT OF CORTICOSTEROID ANALOGUES ON THE RELATIVE THYMUS WEIGHT

Corticosteroid	No. of rats	Total dose per rat mg./100 g.	Mg. thymus/100 g. ± S.E.
Control (maize oil alone)	5	0·0	369·6 ± 18·2
Hydrocortisone	8	0·60	238·4 ± 6·3
	8	1·20	154·5 ± 8·4
20β-Hydroxyhydrocortisone (Reichstein's E)	8	4·79	377·8 ± 12·5
	8	9·58	378·6 ± 10·4
20α-Hydroxyhydrocortisone	6	6·23	370·2 ± 11·4
20α-Hydroxyprednisolone	6	6·27	346·7 ± 14·2
20β-Hydroxyprednisone-21-acetate	8	2·42	382·3 ± 21·8
	7	4·84	376·4 ± 13·8
	7	9·67	367·7 ± 16·6
11β,20α-Dihydroxy-4-pregnene-3-one	6	6·03	402·3 ± 21·7
11β,20β-Dihydroxy-2-methyl-4-pregnene-3-one	6	6·20	361·5 ± 20·0
19-Norhydrocortisone	6	2·34	369·2 ± 15·4
3,11β,17α,21-Tetrahydroxy-19-nor-1,3,5(10)-pregnatriene-20-one	6	2·30	385·7 ± 10·5

test animals at the doses used. These findings suggested therefore that both the C-(20) ketone and the 19-methyl group had a profound effect on the thymolytic potency of hydrocortisone and its analogues.

DISCUSSION

According to Abelson, Ulrich and Long[2], 20β-hydroxyhydrocortisone (Reichstein's Compound E) was the most abundant metabolite found after the administration of hydrocortisone-4-^{14}C to the rat. The monohydrate of 20β-hydroxyhydrocortisone showed activity in the liver glycogen assay in the adrenalectomised mouse, but was less active than hydrocortisone. Szpilfogel, van Hemert, and de Winter[3] reported that the 20β-hydroxy derivatives of both prednisone and prednisolone were active in the liver glycogen deposition test for glucocorticoid activity. However Beyler, Hoffman and Sarett[4] found that 20α-hydroxyprednisone was inactive in the liver glycogen assay. Apparently the 20β-ols had glucocorticoid activity while the 20α-ols did not.

The results obtained in this investigation indicate that neither the 20β-ol nor the 20α-ol derivatives of prednisone or prednisolone were able to produce thymic involution in the intact immature rat at the relatively high doses used.

According to Ehrenstein, 19-nordesoxycorticosterone showed little or no physiological activity when administered at a dose level of 0·3 mg./day to adrenalectomised rats. Removal of the 19-methyl group from hydrocortisone decreased the thymolytic activity to the point where it could not be detected by the assay procedure employed. Likewise substitution of a phenolic type of ring structure for ring A of 19-norhydrocortisone reduced if not abolished the ability of the corticosteroid to involute the thymus gland. Since both testosterone and progesterone retain at least qualitatively their respective biological activities after removal of the 19-methyl

group[6,7], it is concluded that the structure specicificity of the adrenal corticosteroids differs remarkedly from that of the androgens and the progestins.

The results of this study indicate that both the C(20) ketone and the 19-methyl group can be considered to be necessary for thymolytic activity in corticosteroids.

Acknowledgements. The corticosteroids used in this investigation were generously supplied by the Upjohn Co., Lederle Laboratories Division of the American Cyanamid Co., and Merck & Co., Ltd. The author wishes to thank Miss C. A McLeod and Mr. A. J. Bayne for their valuable technical assistance.

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STUDIES IN THE FIELD OF DIURETIC DRUGS

PART II. 5-CHLORO-2,4-DISULPHAMYLTOLUENE (DISULPHAMIDE)

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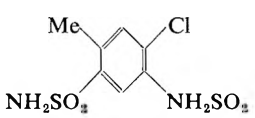
Received March 17, 1960

The preparation of 5-chloro-2,4-disulphamyltoluene, its properties and estimation in biological fluids are described.

IN Part I of this series[1] the preparation and carbonic anhydrase inhibiting activity (CAIA) of some new sulphamyl compounds were reported. Many of these, in particular the esters of *p*-sulphamylbenzoic acid, 4,4'-disulphamyldiphenyl, 4,4'-disulphamyldiphenyl sulphide and the 2-alkoxycarbonylamido-1,2,4-thiadiazoles[2] possessed outstanding activity in the CAIA assay employed. Nevertheless, with the exception of the thiadiazole derivatives, they were virtually inactive as diuretics in the saline-loaded rat. In extending this work we turned to the disulphamyl derivatives of benzene, a group of compounds independently studied in other laboratories[3,4].

Employing standard methods of synthesis, the 1,3-disulphamyl derivatives of benzene listed in Table VI were prepared and screened for carbonic anhydrase inhibiting activity. Study of their diuretic properties (oral administration to the saline-loaded rat) by Dr. A. David and his colleagues[5] led to the selection of 5-chloro-2,4-disulphamyltoluene (I) for further examination.



(I)

5-Chloro-2,4-disulphamyltoluene (disulphamide) (I) is a new compound prepared by chlorosulphonation of *m*-chlorotoluene under regulated conditions, followed by reaction of the resultant 5-chlorotoluene-2,4-disulphonchloride[6] with ammonia. It crystallises from alcohols, aqueous alcohols or glycols in colourless needles or prisms of m.p. 259·5° to 260·5° (corr.). Though possessing identical melting points, the two crystalline forms differ slightly in their infra-red spectra in Nujol suspension. Its pharmacological properties are described elsewhere[5].

EXPERIMENTAL

Solubility

The solubility of disulphamide was accurately determined by equilibrating a saturated solution of the compound in the presence of excess solid for 48 hours in a thermostat bath at 0° and 25°. In the case of the solubility determination at 0° the solution was equilibrated in a refrigerator

fitted with a recording thermometer. The saturated solutions were hand-centrifuged, samples were weighed into volumetric flasks, diluted, and the sulphamide determined by ultra-violet spectrophotometry. Replicate readings showed that the method was comparable in accuracy to the conventional macro-method whilst permitting the use of a sample of about 20 mg. for each determination. The solubilities at 0° and at 25° were found to be 0·02 g. and 0·06 g. per 100 g. of water, respectively.

Disulphamide is soluble to about 2 per cent in boiling water and is readily soluble in aqueous alkali hydroxides or carbonate.

TABLE I

SOLUBILITY OF 5-CHLORO-2,4-DISULPHAMYLTOLUENE IN AQUEOUS ETHANOL AT 25°

Ethanol (per cent by volume)	Solubility (g. solute per 100 g. of solvent)
100	1·89
90	2·23
80	2·41
70	2·08
60	1·44
50	1·17
40	0·63
20	0·15

The solubility in water-ethanol at 25° reached a maximum at about 80 per cent (v/v) of ethanol (Table I).

The solubility of the compound in other organic solvents at 25° (g. solute per 100 g. solvent) was as follows: hexane (0·0004); chloroform (0·001) and isopropanol (0·35).

Ultra-violet Absorption Spectra

Ultra-violet absorption spectra were determined using a Beckman recording spectrophotometer, Model DK-2. The results obtained in

TABLE II

ULTRA-VIOLET ABSORPTION SPECTRA OF 5-CHLORO-2,4-DISULPHAMYLTOLUENE IN VARIOUS SOLVENTS

Solvent	λ max (mμ)	ε max
Isopropanol	285	920
	276	1,035
	235	12,550
Ethanol	285	805
	276	897
	235	11,930
	210	46,000
50 per cent ethanol	285	957
	276	1,050
	235	11,790

isopropanol, ethanol and 50 per cent (v/v) aqueous ethanol are summarised in Table II. Acidification of a 1 : 1 aqueous-ethanolic solution to 0·05N with hydrochloric acid had no effect upon the spectral absorption. Addition of potassium hydroxide (0·05N) completely eliminated all spectral detail and the curve rose steadily from *ca.* 295 mμ to a maximum

at 221 mμ (εmax = 17,800). Thereafter there was a shallow minimum at 220 mμ when the curve rose again to an arbitrarily chosen lower wavelength limit of 218 mμ. These observations indicate a simple acid-base reaction between relatively stable species, the spectra in acid and alkali being mutually interconvertible by excess reagent on equilibration.

Determination of 5-Chloro-2,4-Disulphamyltoluene in Biological Fluids

Determination of disulphamide was carried out by measurement of the carbonic anhydrase inhibiting activity, using the procedure described

TABLE III

5-Chloro-2,4-disulphamyltoluene found in urine of four groups of 10 rats dosed with 300 mg./kg. (total dose = 3·2 g.) of the diuretic

Urine		5-Chloro-2,4-disulphamyl toluene found (mg.)	
Time (hours)	Volume (ml.)	By enzyme inhibition	By isolation
0–2	200	64	76
2–4	130	203	138
4–6	62	54	92
6–24	100	199	—

previously[1]. This method is known to have an error of ±50 per cent. A similar assay has previously been described for the assay of acetazolamide[7].

Preparation of Biological Samples for Assay

Urine samples. These were acidified with hydrochloric acid and extracted three times with ethyl acetate. The ethyl acetate solutions were then extracted three times with 5 per cent sodium carbonate solution, and residual ethyl acetate was removed from the aqueous solution by aeration. The solutions were made up to 200 ml. with 5 per cent sodium carbonate solution, and a minimum dilution of this solution of 1 in 100 with 50 per cent ethanol was taken for assay.

Blood samples. These were separated into cells and serum, each being made up to a 1 in 10 dilution with normal saline, and further 1 in 5 dilutions were made with distilled water. These solutions were heated for 5 minutes in a boiling water bath to destroy endogenous enzyme activity. Not more than 0·8 ml. of these solutions could be used in the assay procedure, since other constituents caused interference if larger quantities were taken. Recovery of material added to normal specimens was within experimental error.

Added to urine: 7·6 mg./l. Recovered: 9·9 mg./l. Added to 1 in 50 cell dilution: 0·24 mg./l. Recovered: 0·38 mg./l. Added to 1 in 50 serum dilution: 0·24 mg./l. Recovered: 0·34 mg./l.

Standards for the assay of disulphamide in biological specimens were prepared by addition of the compound to normal biological specimens. Both sets were then prepared for assay as described above.

Determination of the nature of the inhibitory activity in urine from dosed animals. Forty rats were dosed orally with 300 mg./kg. of a drug suspension, and their urine was subjected both to the assay procedure and to direct isolation of the drug. The drug was isolated by the following procedure. The urine was acidified, extracted three times with ethyl acetate, and the bulked ethyl acetate extract was extracted three times with sodium carbonate solution. Acidification of the concentrated carbonate solution led to the formation of crystals which were filtered off on a sintered crucible, dried, and weighed. Identity was established by determination of mixed melting point with an authentic sample. Results obtained are

TABLE IV

5-CHLORO-2,4-DISULPHAMYLTOLUENE FOUND IN URINE OF 8 RATS DOSED WITH 10 MG./KG. OF THE DIURETIC

5-hour volume of urine (ml.)	Total dose administered (mg.)	Recovery	
		mg.	per cent of dose
16	22·75	5·04	22·2
19	22·25	5·76	25·3
25	25·44	5·85	23·0
23·6	23·10	7·43	32·2
24·2	22·75	11·01	48·4

listed in Table III. Agreement was sufficiently close, within the limits of experimental error, to warrant the conclusion that the measured carbonic anhydrase inhibitory activity was due to the presence of unchanged 5-chloro-2,4-disulphamyltoluene.

Recovery of dose from urine. In the above high-dose experiment approximately 15 per cent of the dose was accounted for as being excreted unchanged in the urine. In a second experiment five groups of 8 rats were given an oral dose of 10 mg./kg., and 20 to 50 per cent of the dose was excreted in 5 hours. Results are listed in Table IV.

TABLE V

5-CHLORO-2,4-DISULPHAMYLTOLUENE IN SERUM AND RED CELLS OF RABBITS RECEIVING 10 MG./KG. OF THE DIURETIC BY MOUTH

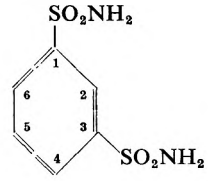
Hours	5-Chloro-2,4-disulphamyltoluene in serum (μg./ml.)		5-Chloro-2,4-disulphamyltoluene in red cells (μg./ml.)	
	Rabbit A	Rabbit B	Rabbit A	Rabbit B
1	<0·5	2·3	<0·5	3·6
2	0·9	1·7	1·3	1·4
4	<0·5	<0·5	2·1	0·6
6	<0·5	0·7	1·7	<0·5
24	<0·5	<0·5	<0·5	<0·5

Distribution of drug in blood. Two rabbits were given an oral dose of 10 mg./kg., and blood samples were taken periodically and assayed in serum and red cells. Results obtained are given in Table V.

5-Chlorotoluene-2,4-disulphonchloride. m-Chlorotoluene (126·5 g.) was added dropwise with stirring to chlorosulphonic acid (350 g.), the rate of addition being controlled to keep the temperature of the reaction below

TABLE VI

DISULPHONAMIDES



Substituent at position			CA* activity (w/w) acetazolamide = 1	m.p. °C.	Formula	Found					Required				
4	5	6				C	H	N	S	Cl	C	H	N	S	Cl
—	—	—	0·01	233–235											
Me	—	—	0·30	189–190	$C_7H_{10}O_4N_2S_2$	34·0	3·8	10·8	25·6	—	33·6	4·0	11·2	25·6	—
Et	—	—	0·04	185–187	$C_8H_{12}O_4N_2S_2$	36·6	4·3	10·6	24·0	—	36·4	4·6	10·6	24·3	—
Me_2CH	—	—	0·04	213–214	$C_9H_{14}O_4N_2S_2$	39·2	5·2	9·7	—	—	38·8	5·1	10·1	—	—
F	—	—	0·10	189–191	$C_6H_7O_4N_2S_2F$	28·6	2·4	11·0	—	—	28·3	2·8	11·0	—	—
Cl	—	—	0·09	212–214	—	—	—	—	—	—	—	—	—	—	—
Br	—	—	0·06	243–245	—	—	—	—	—	—	—	—	—	—	—
Me	Me	—	0·25	237–239	—	—	—	—	—	—	—	—	—	—	—
Me	—	Me	0·47	257–259	—	—	—	—	—	—	—	—	—	—	—
Me	—	F	0·28	216–218	$C_7H_9O_4N_2S_2F$	31·6	3·3	10·5	23·4	—	31·4	3·4	10·5	23·9	—
Me	—	Cl	0·40	259·5–260·5 corr.	$C_7H_9O_4N_2S_2Cl$	29·9	2·9	9·9	—	—	29·5	3·2	9·8	22·5	12·5
Me	Cl	—	2·00	237–239	$C_7H_9O_4N_2S_2Cl$	29·9	3·2	10·1	22·3	12·6	29·5	3·2	9·8	22·5	12·5
Cl	Me	—	0·67	239–240	$C_7H_9O_4N_2S_2Cl$	30·1	3·2	9·8	22·7	12·5	29·5	3·2	9·8	22·5	12·5
Me	—	Br	0·43	264–265	$C_7H_9O_4N_2S_2Br$	25·8	2·8	8·2	—	—	25·5	2·7	8·5	—	—
Et	—	Cl	0·56	194–195	$C_8H_{11}O_4N_2S_2Cl$	32·1	3·4	9·4	21·5	11·9	32·2	3·7	9·4	21·5	11·9
n–Pr	—	Cl	0·55	179–180	$C_9H_{13}O_4N_2S_2Cl$	34·3	3·8	8·9	20·9	11·7	34·6	4·2	9·0	20·5	11·4
Cl	Cl	—	2·00	234–236	$C_6H_6O_4N_2S_2Cl_2$	—	—	—	—	—	—	—	—	—	—
Cl	—	Cl	0·66	265	$C_6H_6O_4N_2S_2Cl_2$	23·4	2·3	9·6	20·7	23·0	23·6	2·0	9·2	21·0	23·3
Br	—	Br	0·40	271–273	$C_6H_6O_4N_2S_2Br_2$	18·7	1·8	7·2	15·8	44·4†	18·3	1·5	7·1	16·2	40·5†
Cl	—	F	0·40	234–235	$C_6H_6O_4N_2S_2ClF$	25·0	2·2	9·7	—	11·8	25·0	2·1	9·7	—	12·3
Cl	—	Br	0·32	290	$C_6H_6O_4N_2S_2ClBr$	20·9	1·9	—	—	—	20·7	1·7	—	—	—
$-+$ ONH_4	Cl	—	0·0002	252 decomp.	$C_6H_{10}O_5N_3S_2Cl$	23·9	3·2	13·5	—	11·6	23·7	3·2	13·8	—	11·7
$-+$ ONH_4	SO_2NH_2	—	<0·0002	266 decomp.	$C_6H_{13}O_7N_4S_3$	21·2	3·1	16·1	27·7	—	20·7	3·5	16·1	27·6	—
Cl	SO_2NH_2	—	0·17	344 decomp.	$C_6H_8O_6N_3S_3Cl$	20·9	2·6	11·6	27·8	10·0	20·6	2·8	12·0	27·5	10·1

* Carbonic Anhydrase.

† Bromine.

50°. After the addition was complete, pentachloroethane (250 ml.) was added to the stirred mixture which was then gradually heated to reflux temperature. The heating was continued until evolution of hydrogen chloride was complete (usually about 8 hours). The mixture was then heated on the steam bath at about 20 mm. pressure to remove all volatile material. The hot residue was extracted with a high boiling (100–120°) light petroleum solvent, two extractions usually being sufficient. The *product* separated from the light petroleum extract on cooling; it had m.p. 122–124°, and was sufficiently pure for the next stage of the process. A portion, crystallised from ligroin, had m.p. 125–126°. Found: S, 20·1; Cl, 32·8. Calc. for $C_7H_5O_4S_2Cl_3$: Cl, 32·9; S, 19·8 per cent.

5-*Chlorotoluene*-2,4-*disulphonamide*. The foregoing disulphonchloride (100 g.) was added in portions with stirring to liquid ammonia and when the addition was complete, excess of ammonia was allowed to evaporate at room-temperature. The semi-solid residue was dissolved in water (500 ml.) and the solution acidified with hydrochloric acid. The *product* which separated had m.p. 257–259°. The m.p. was 259·5–260·5° (corr.) after crystallisation from aqueous ethanol.

Fluorobenzene-2,4-*disulphonchloride*. A mixture of fluorobenzene-4-sulphonchloride (150 g.) and chlorosulphonic acid (300 ml.) was heated at 160–170° for 20 hours. The cooled mixture was poured on to ice and the *product* isolated with carbon tetrachloride and purified by distillation under reduced pressure. It had b.p. 132–139° at 0·5 mm., and m.p. 69–71° after crystallisation from light petroleum (b.p. 60–80°). Found: C, 24·9; H, 1·3; Cl, 24·5. $C_6H_3O_4S_2Cl_2F$ requires C, 24·6; H, 1·0; Cl, 24·2 per cent.

5-*Fluorotoluene*-2,4-*disulphonchloride*, prepared by chlorosulphonation of 3-fluorotoluene in pentachloroethane, had m.p. 99–100° after crystallisation from ligroin. Found: S, 21·3; Cl, 22·8. $C_7H_5O_4S_2Cl_2F$ requires S, 20·9; Cl, 23·1 per cent.

5-*Bromotoluene*-2,4-*disulphonchloride*, crystallised from ligroin in white needles, m.p. 121–122°. Found: C, 22·8, H, 1·4; S, 17·1; Br, 21·9; Cl, 19·4; $C_7H_5O_4S_2Cl_2Br$ requires C, 22·8; H, 1·4; S, 17·4; Br, 21·7; Cl, 19·3 per cent.

5-*Chlorofluorobenzene*-2,4-*disulphonchloride*, had m.p. 103–105° after crystallisation from ligroin. Found: C, 22·4; H, 0·9; Cl, 32·1. $C_6H_2O_4S_2Cl_3F$ requires C, 22·0; H, 0·6; Cl, 32·5 per cent.

5-*Bromochlorobenzene*-2,4-*disulphonchloride*, had m.p. 139–141° after crystallisation from ligroin. Found: C, 18·9; H, 0·9; S, 16·6. $C_6H_2O_4S_2Cl_3Br$ requires C, 18·6; H, 0·5; S, 16·5 per cent.

2,4-*Dibromobenzenesulphonchloride*, was obtained as a low melting solid of b.p. 128° at 0·2 mm. by chlorosulphonation of *m*-dibromobenzene (129 g.) with chlorosulphonic acid (200 g.) in carbon tetrachloride (125 g.) at reflux temperature for 16 hours. Found: C, 21·7; H. 1·0. $C_6H_3O_2SClBr_2$ requires C, 21·6; H, 0·9 per cent.

4,6-*Dibromobenzene*-1,3-*disulphonchloride*, was obtained by heating the foregoing compound (100 g.) with chlorosulphonic acid (150 ml.) at 150° for 20 hours. It had m.p. 159–162° after crystallisation from ligroin.

Found: C, 17·0; H, 0·7; S, 14·5. $C_6H_2O_4S_2Cl_2Br_2$ requires C, 16·6; H, 0·5; S, 14·8 per cent.

4-*Chloro*-3,5-*xylenol*-2,6-*disulphonamide*, prepared by chlorosulphonation of 4-chloro-3,5-xylenol at room temperature followed by reaction of the resultant disulphonchloride with liquid ammonia, had m.p. 242–244° after crystallisation from aqueous ethanol. Found: C, 31·0; H, 3·7; N, 9·2; Cl, 11·5. $C_8H_{11}O_5N_2S_2Cl$ requires C, 30·6; H, 3·5; N, 8·9; Cl, 11·3 per cent.

Other disulphonamides prepared by methods already illustrated are summarised in Table VI.

Acknowledgements. The authors thank Dr. A. David for providing the blood and urine samples.

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A NOTE ON THE INFLUENCE OF ULTRASONIC VIBRATIONS ON PYROGEN MATERIALS IN DISTILLED WATER

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Received March 7, 1960

Pyrogenic distilled water and an autoclaved standard suspension of *B. subtilis* lost their pyrogenic activity when treated with ultrasonic vibrations.

No reference to the effect of ultrasonic vibrations on pyrogen materials from distilled water has appeared in the literature. It is accepted that pyrogens are bacterial residues or products of bacterial metabolism[1] containing nitrogen and that they may be either proteins or polysaccharides[2]. Studies of the effect of ultrasonic vibrations on the structure of polysaccharides and proteins[3,4] has shown that the changes of molecular structure could be induced. Therefore we have attempted to change the structure of pyrogens by this means with the object of destroying their pyrogenic activity.

Strongly reacting and pyrogen negative solutions were chosen for treatment. The pyrogen tests were made according to the requirements of the U.S.P. XV. 50 ml. batches of distilled water were placed in a heavy open glass cylinder 30 mm. in diameter and 25 cm. in length. The bottom of the cylinder was closed with a rubber membrane, 45μ thick, and the top with a glass cover. The rubber membrane was placed directly on the quartz crystal of a suitable generator. The system was placed in a water bath with constant temperature (18°) to avoid the effects of heating.

Solutions were exposed to vibrations under the following conditions:

1. Frequency: 800 kc./sec.
   Intensity: 1·5 W/cm.^{2}
   Duration: 5 and 10 min.
2. Frequency: 2 Mc./sec.
   Intensity: 2 W/cm.^{2}
   Duration: 10 min.

One group of rabbits received pyrogenic distilled water as an isotonic solution. A second group received the same solution previously treated with ultrasonic vibrations at 2 Mc./sec. Figure 1 shows the temperature changes of the two groups.

As can be seen, the control group showed a pyrogenic reaction, and the animals which received the pretreated solution did not react. The difference was found to be significant.

A frequency of 800 kc./sec. and intensity of 1·5 W/cm.^{2} for both, 5 and 10 minutes, produced a diminution in the pyrogenic reaction but it was not significant.

All animals which were injected with the treated distilled water did not show any change in temperature, appetite and general condition in the 30 days after injection.

The pH and refractive index of distilled water were measured before and after treatment. No change in refractive index was observed. The

treatment of pyrogenic distilled water caused a fall in pH from 6·75–6·80 to 6·20–6·10.

To compare the results obtained on pyrogenic distilled water with those obtained from a known pyrogenic system, a standard suspension of *B. subtilis* was added to apyrogenic distilled water and autoclaved at 120° for 30 minutes. The resulting solution gave a definite pyrogen

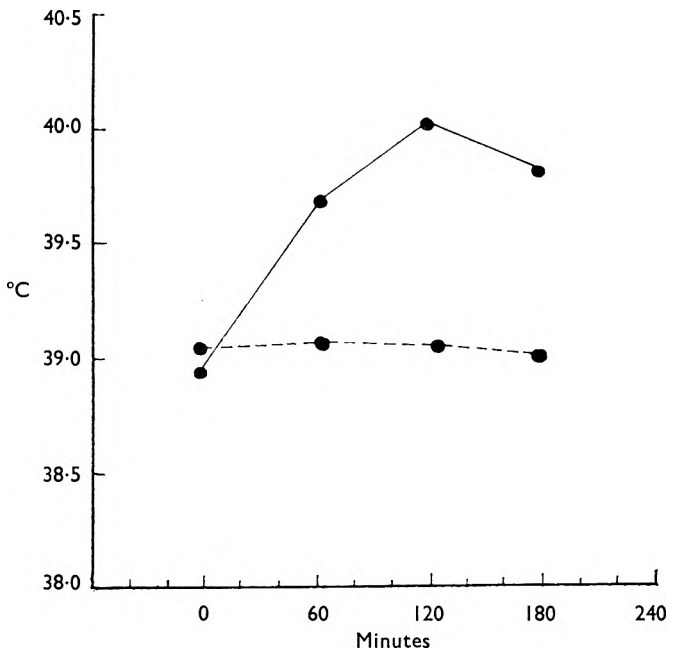

FIG. 1. Temperature changes in rabbits injected with ●—● untreated pyrogenic distilled water and ●---● distilled water treated at a frequency of 2 Mc./sec. intensity 2 W/cm.² for 10 min.

reaction. After treatment by 2 Mc./sec. the solution gave no pyrogen reaction in four groups of rabbits. 800 kc./sec. produced a reaction lower than the untreated solution. These results are parallel to those obtained with pyrogenic distilled water.

We conclude that the pyrogenic distilled water lost its pyrogenic activity when treated. Similarly, an autoclaved standard suspension of *B. subtilis* lost its pyrogenic activity.

Therefore it is possible that the vibrations have an application both in the production of apyrogenic distilled water and in the study of these ubiquitous substances. Further work is in progress and a full report will be published later.

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EFFECTS OF GIBBERELLIC ACID ON THE GROWTH AND ALKALOIDAL CONTENT OF *DATURA STRAMONIUM* L.

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Received March 10, 1960

Gibberellic acid, at three dose levels, was injected into growing plants of *Datura stramonium* and the effects on plant habit, yield, and total alkaloidal content of the leaves and tops recorded. Increase in height of treated plants was due to increased internode length, especially on the treated side of the plant. Rapid growth in stem diameter caused axial splitting of the outer tissues resulting in the appearance of scar tissue. Increases in dry weights of aerial parts, observed only in plants treated over a limited period, were due to greater stem development and did not vary with the dose of gibberellic acid given. In plants treated at intervals over a long growing period, there was increased stem production but no gain in total dry weight. Yields of leaves and tops in treated plants were not significantly different from those in control groups. Significantly increased root weights were noted for a few groups of treated plants but proportion of root to total plant weight remained constant. There was a significant decrease in percentage alkaloidal content of leaves and tops in treated plants, the content being lowest in the plants which had received the highest dose of gibberellic acid

Some effects of gibberellic acid on the growth and alkaloidal content of *Datura stramonium*, *Atropa belladonna*[1,2], and *Hyoscyamus niger*[3] have been reported. In the first paper results were described for plants of *Datura stramomium* grown under greenhouse conditions and treated over a short growth period. In the present communication results are given for plants of this species grown under field conditions and treated with successive doses of gibberellic acid over intermediate and long growth periods.

Experimental

In 1958, plants of *D. stramonium* were raised from a single strain of seeds obtained from Chelsea Physic Garden and in 1959 from seeds taken from a single capsule of an untreated plant grown in the previous season. Seeds were sown in March, the seedlings raised in a heated greenhouse and hardened off in a cold frame before transplanting into well worked soil in mid-June. In 1958, 100 strong plants were arranged in rows of 10, allowing 18 inches between each plant, in a plot of virgin soil to which a balanced fertiliser had been added at the rate of 200 g. per square yard. The plants were left for 1 week to establish before treatment began, then each plant was randomly assigned to one of ten groups which were treated as follows. Two groups (G and H) received 20μg., two groups (D and J) 100 μg., and two groups (A and C) 200 μg. gibberellic acid per dose per plant.

The gibberellic acid was dissolved in absolute ethanol sufficient to give the required dose in 0·002 ml. and this was injected by means of a micrometer syringe into the base of a leaf situated near the growing point on one

side of the plant. Five such treatments were given at weekly intervals. Four groups of plants (F, I, B, and E) were left as untreated controls. In each of the groups C, J, and I, one plant became stunted in growth after transplanting: these were not included in the experiment. Flowers were removed from all plants at intervals, so that no fruits were allowed to develop, since it has been shown in species of *Datura* that removal of the

FIG. 1. *Datura stramonium* L., untreated plant, x 1/8. Scale = 10 cm.

flowers leads to increased alkaloidal content in the leaves[4,5], whereas at the time of fruit and seed formation there is a gradual decrease in the amount of leaf alkaloid[6].

The first collection, of entire plants in groups G, D, A, and F, was made 1 week after the final treatment; the second collection, plants of groups H. J. C, and I, 2 weeks after the final treatment; and the third collection of untreated plants in groups B and E, was made after a further 5 weeks. Collection was made early in the day during dry weather. Each plant was handled separately, the roots carefully removed, washed, dried, and weighed. The leaves and tops were separated from the stouter main stems and branches, and the materials dried in an air oven at 55°, then weighed. In Stramonium B.P. there is a limit for the amount of stems exceeding 5 mm. width, therefore the leaves and tops only were prepared for assay by powdering, and sifting through a No. 60 sieve. The samples

were stored in well filled, screw-capped jars containing silica gel as a desiccant.

Having observed that any increased weight due to gibberellic acid treatment was negligible compared with increases resulting from extended periods of growth, it was decided to treat plants with gibberellic acid at intervals throughout the growing season to see if the stimulant effect could be prolonged. In 1959, 42 young plants were set out in a different plot from that used in 1958 and 50 g. of balanced fertiliser was worked into the soil around each plant. The plants were randomly allocated to three groups. After 1 week the following treatments were started: plants in group Y received 20 μg. doses and plants in group Z, 200 μg. doses of gibberellic acid at weekly intervals for 4 weeks, followed by three similar doses injected at fortnightly intervals. Ten days after the final injection all plants, including the untreated controls (group X), were collected at the same time, measured for height and width of aerial parts (Table I), then divided, dried, and weighed as before. The leaves and tops were powdered and assayed.

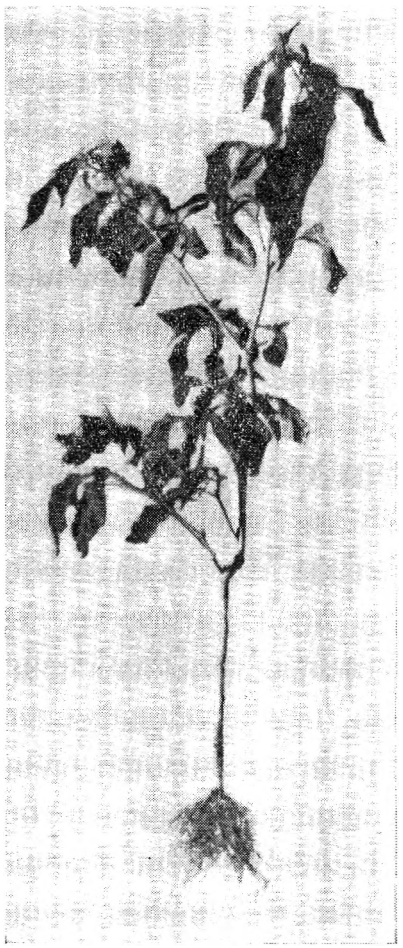

FIG. 2. *Datura stramonium* L., plant treated with gibberellic acid showing reduction in the angle of branching, x 1/8.

Assay

Samples were assayed for total alkaloidal content using the Vitali colour reaction and following the method of Jackson[7], with slight modifications.

One g. of powder was weighed into a 30 ml. beaker and stirred with 0·9 ml. of ethanol (95 per cent) and 0·1 ml. of dilute solution of ammonia. Five ml. of chloroform was added and the mixture heated just to boiling; it was then transferred to a miniature percolator, washing out the beaker with further quantities of boiling chloroform. Percolation was continued with cold chloroform until exactly 31 ml. percolate had collected. Sufficient 6 per cent solution of acetic acid (made with 5 per cent ethanol) was

added to the percolate to bring the volume to exactly 80 ml. The mixture was shaken gently for 30 seconds, allowed to separate, and 5 to 10 ml. of the acid layer filtered through dry filter paper. 0·5 ml. of the filtrate was evaporated just to dryness in a small dish over a boiling water bath, the residue thoroughly moistened with 0·2 ml. of fuming nitric acid (Analar S.G. 1·5) and immediately heated to dryness over a water bath. The nitrated residue was dissolved in about 6 ml. acetone (Analar) and transferred to a dry 10 ml. volumetric flask; 2 ml. of isopropylamine[8], then

TABLE I

EFFECT OF GIBBERELLIC ACID ON PLANT HEIGHT AND WIDTH

Group	No. of plants	Treatment	Height (cm.)	Max. width (cm.)
X	14	Controls	43 – 52·3 – 70	51 – 63·5 – 79
Y	14	7 × 20 μg. acid per plant	69 – 78·2 – 104	36 – 59·9 – 91
Z	14	7 × 200 μg. acid per plant	66 – 87·1 – 99	33 – 52·8 – 81

0·1 ml. of a freshly prepared 2 per cent solution of potassium hydroxide (Analar) in absolute methanol were added and the volume made up to exactly 10 ml. with acetone. The colour density of the solution was measured at a wavelength of 555 mμ in a 1 cm. cell of a Unicam SP. 600 spectrophotometer, using acetone as a blank, exactly 2 minutes after the addition of the potassium hydroxide since the colour is not stable. The

TABLE II

EFFECT OF GIBBERELLIC ACID ON PLANT DRY WEIGHT YIELD

				Aerial parts		Leaves and tops		Stems		Roots	
	Group	No. of plants	Treatment per plant	Mean wt. (g.)	*t*	Mean wt. (g.)	*t*	Mean wt. (g.)	*t*	Mean wt. (g.)	*t*
1st Crop 1958	F	10	Controls	10·18		8·40		1·78		1·70	
	G	10	5 × 20 μg. acid	10·51	0·21	7·96	0·34	2·55	1·90	1·63	0·27
	D	10	5 × 100 μg. ,,	12·66	1·58	9·53	0·88	3·13	3·32	2·34	2·46
	A	10	5 × 200 μg. ,,	10·38	0·13	8·02	0·30	2·36	1·43	2·02	1·23
2nd Crop 1958	I	9	Controls	13·94		10·99		2·95		2·52	
	H	10	5 × 20 μg. acid	18·66	2·08	11·71	0·57	6·95	3·86	3·16	3·11
	J	9	5 × 100 μg. ,,	18·10	1·78	11·02	0·32	7·08	3·89	3·48	4·55
	C	9	5 × 200 μg. ,,	18·86	2·11	11·61	0·50	7·25	4·05	3·30	3·70
3rd Crop 1958	B	10	Controls	70·19		47·27		22·92		11·15	
	E	10	,,	68·86		46·30		22·56		11·30	
1959 Crop	X	14	Controls	28·71		24·01		4·70		5·03	
	Y	14	7 × 20 μg. acid	28·57	0·02	20·63	1·31	7·94	2·44	6·84	2·21
	Z	14	7 × 200 μg. ,,	27·63	0·29	18·20	2·26	9·43	3·57	6·31	1·56

For P = 0·95, *t* = 2·04.

alkaloidal content of each sample was calculated from the means of duplicate or triplicate readings, using a conversion factor (K) determined from colour density figures obtained using 1 to 5 ml. quantities of 0·1 per cent chloroformic solution of atropine in place of the 1 g. samples of powder in the previously described method. $K = \frac{\text{colour density}}{\text{mg. atropine}} = 8{\cdot}2.$

The content of alkaloid per cent was calculated with reference to each sample dried to constant weight at 105°.

RESULTS

Effects of Gibberellic Acid on Morphology of Plants

All treated plants were taller than untreated controls (Fig. 1) due to greater stem development, but they were usually less spreading due to a reduction in the angle of branching (Fig. 2). Measurements of heights

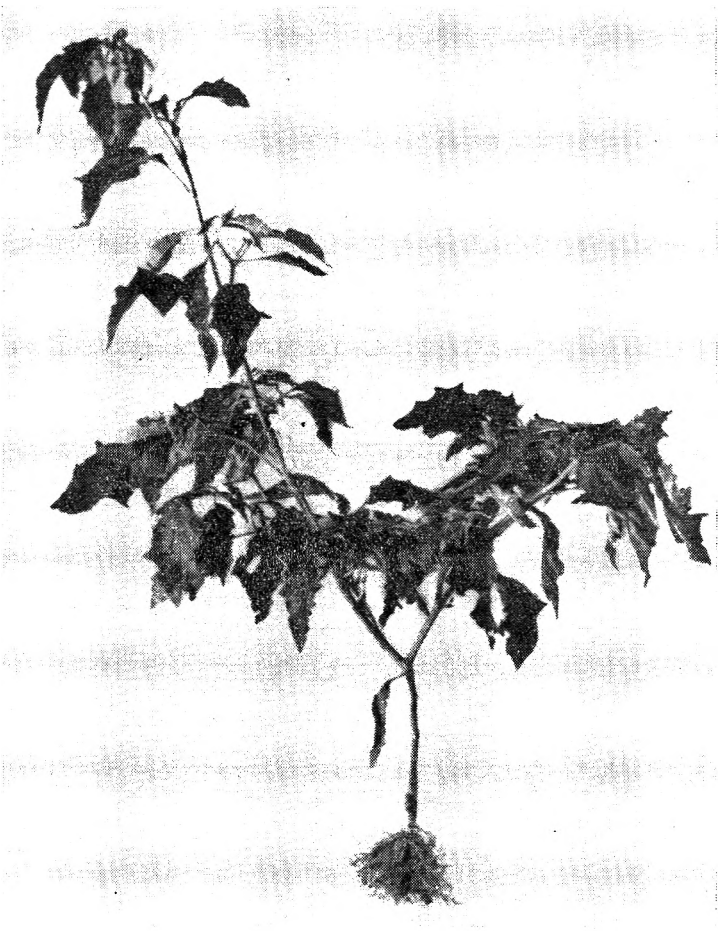

FIG. 3. *Datura stramonium* L., plant treated with gibberellic acid on one side only, x 1 8.

and maximum widths for the 1959 crop, at the time of collection, are given in Table I. It was noticeable that plants injected on the same side, relative to the branching at the first node, exhibited the characters of a treated plant only on the treated side, the appearance on the non-treated

side being normal (Fig. 3). The stems on the treated side were thicker, and on the internode beyond the first fork numerous closely-arranged, axially elongated, pale brown areas of scar tissue were always present (Fig. 4). Frequently, similar but fewer, scars appeared on the higher internodes of this side; sometimes scars appeared on the main stem below

FIG. 4. *Datura stramonium* L. Scar tissue on stem of plant treated with gibberellic acid, x 1/2.

the first node, but only rarely were a few small scars observed on the non-treated side and, if present, they were very close to the first node. The scarring was due to vertical splitting of the outer tissues, often all tissues external to the xylem, as a result of rapid increase in stem width, the wounds being subsequently closed by scar tissue.

Leaves on the treated side were paler in colour, many were narrower, though not longer, than normal leaves and the margin often showed a much reduced indentation. Plants receiving the largest dose of gibberellic acid were usually the least healthy in appearance.

Effect of Gibberellic Acid on Yield of Plant Material

Table II shows the mean values for weights of total aerial parts, leaves and tops, stems, and roots. The values for t were calculated using figures obtained in analyses of variance on the weights for individual plants in each treated group compared with the weights of plants in control groups collected at the same time.

TABLE III

EFFECT OF GIBBERELLIC ACID ON ALKALOIDAL CONTENT OF LEAVES AND TOPS IN INDIVIDUAL PLANTS

Plant No.	Total alkaloid per cent				
	Group I controls	Group H 5 × 20 μg. acid per plant	Group J 5 × 100 μg. acid per plant	Group C 5 × 200 μg. acid per plant	Group B controls (late collection)
1	0·559	0·476	0·414	—	0·510
2	0·452	0·488	0·362	0·422	0·519
3	0·524	0·412	0·435	0·351	0·506
4	—	0·427	—	0·302	0·633
5	0·539	0·385	0·398	0·418	0·464
6	0·485	0·483	0·503	0·388	0·567
7	0·569	0·464	0·385	0·388	0·709
8	0·555	0·401	0·481	0·396	0·656
9	0·639	0·448	0·407	0·415	0·715
10	0·532	0·491	0·495	0·372	0·627
Mean	0·539	0·447	0·431	0·384	0·591
t		3·78	4·33	6·21	0·53

For P = 0·95, t = 2·04 except in comparison of Groups I and B where for P = 0·95, t = 2·31.

TABLE IV

EFFECT OF GIBBERELLIC ACID ON TOTAL ALKALOIDAL CONTENT OF LEAVES AND TOPS, DETERMINED ON POOLED SAMPLES

	Group	No. of plants	Treatment per plant	Alkaloid per cent
1st Crop 1958	F	10	controls	0·403
	G	10	5 × 20 μg. acid	0·330
	D	10	5 × 100 μg ,,	0·272
	A	10	5 × 200 μg. ,,	0·298
2nd Crop 1958	I	9	controls	0·537
	H	10	5 × 20 μg. acid	0·492
	J	9	5 × 100 μg. ,,	0·449
	C	9	5 × 200 μg. ,,	0·385
3rd Crop 1958	B	10	controls	0·597
	E	10	,,	0·631
1959 Crop	X	14	controls	0·809
	Y	14	7 × 20 μg. acid	0·685
	Z	14	7 × 200 μg. ,,	0·577

It can be seen that only plants of the second collection in 1958 showed significant increases in total weight of aerial parts as compared with controls, but most groups of treated plants showed significant increases in stem weight. The groups with increased total weight yielded approximately the same weight of leaves and tops as controls. Other groups, including those treated over a prolonged period in 1959, showed decrease in weight of leaves and tops but the only statistically significant reduction was found in plants which received the high dose of gibberellic acid in

1959 (Group Z). The late collected controls of the 1958 crop showed great weight increases over all groups collected earlier, but it is interesting to note that the proportion of stem to total weight in these older plants, while higher than in the early collected controls, was lower than in any group of treated plants.

The effect on root weight was variable, there being significant increases in some, but not all, treated groups in the three crops examined. However the ratio of root weight to total plant weight remained fairly constant.

Effect of Gibberellic Acid on Total Alkaloidal Content of Leaves and Tops

For groups C, J, H, I, and B the leaves and tops from each plant were assayed separately and the results (Table III) treated statistically. The results for the first four groups were analysed for variance and the value of *t* calculated. A significant reduction in alkaloidal content was observed in each group of treated plants, as compared with the control group collected at the same time, the content falling with increase in dose of gibberellic acid given. The percentage of alkaloid was not significantly different in the two groups of untreated plants although the total production per plant was much greater in the late collected controls (Group B) since these showed a considerable increase in leaf yield.

For these and the remaining groups pooled samples of leaves and tops were assayed and the results are given in Table IV. It was again evident that gibberellic acid treatment had caused a loss in alkaloid yield.

Discussion

Increases in plant height, due to increased internode length, and chlorosis of leaves resulting from gibberellic acid treatment are well known for many plants, including *D. stramonium*[1], and these effects have been confirmed for this species.

By injecting this growth-promoting substance the dosage could be carefully controlled and the site of treatment selected, two advantages as compared with the application of an aqueous spray. It is known that gibberellic acid is quickly translocated from the point of application to other parts of the plant, but it is of interest to note that in this dichasially branched species only the treated side of the plant appeared to be affected. Splitting of the outer stem tissues was an effect of treatment and from these observations it appears that gibberellic acid passes from the apex of the treated stems down to the main stem, but not across to the untreated side of the plant.

Dry weight figures confirm that gibberellic acid treatment causes increased stem production but the present results conflict with an earlier report[1] that treatment gives increases in yield of leaves and tops and a decrease in root weight for stramonium. It appears that total weights of aerial parts can be increased by a limited number of treatments with gibberellic acid but further doses do not continue the stimulus to increased growth throughout the growing season.

Confirmation is given that treatment causes significant reduction in total alkaloidal content of leaves and tops[1]. The higher the dose of

gibberellic acid given the greater the effect; the lower doses caused about 20 per cent reduction and the higher doses about 30 per cent reduction in the proportion of alkaloid present, as compared with untreated controls.

Acknowledgements. The author wishes to thank Plant Protection Ltd. and I.C.I. Ltd. (Pharmaceuticals Division) for gifts of gibberellic acid; Mr. E. W. Curtis, Curator of the Botanic Gardens, Glasgow for raising the seedlings used in this work; Mr. J. C. Eaton for advice on the statistical treatment of results; and Miss Agnes Bradley for technical assistance.

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NEW APPARATUS

A CONTINUOUS EXTRACTOR FOR USE IN TOXICOLOGICAL ANALYSIS

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Received February 26, 1960

A continuous extractor using ethanol under reduced pressure has been developed for the isolation of many poisons from viscera. Barbiturates, alkaloids and glycosides have been investigated.

In the many processes suggested over the last hundred years for the extraction of poisons that are soluble in organic solvents, ethanol has figured as the solvent most used in the initial extraction scheme. The original method Stas and Otto has been modified by many workers and is still used exclusively in many countries. Notwithstanding the fact that the process is lengthy and somewhat tedious it must be used in parts of the world where, because of the high temperatures, ethanol is used as a

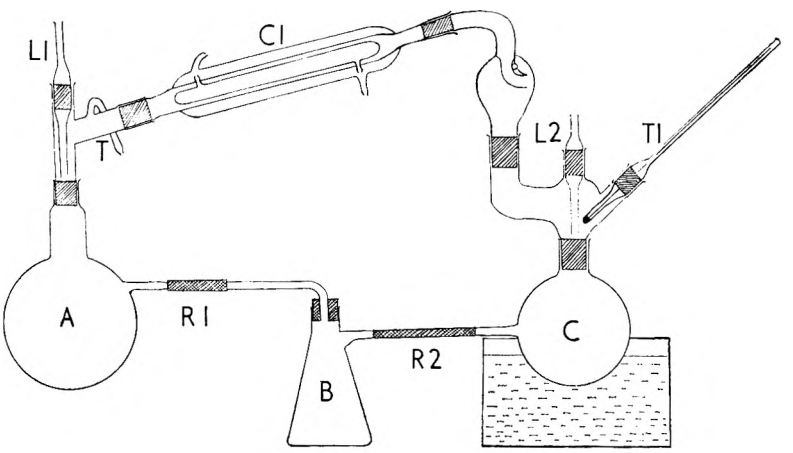

Fig. 1.

preservative. The alternative methods for the extraction are therefore not available to these toxicologists. Although Umberger[1] has described a method using continuous extraction of tissue with ethanol, unfortunately his device is complicated and not readily available commercially. We have therefore developed the apparatus shown in Figure 1. It is a continuous extractor which works under reduced pressure and uses ethanol as the extracting solvent. The tissue, liver, brain or contents of the alimentary tract, is macerated in a blender with 95 per cent ethanol and placed in the large flask "A" of either 1 or 2 litre capacity. The 1 litre extraction flask conveniently handles 100 to 150 g. samples of tissue. The 2 litre flask is adequate for 500 g. samples.* The ethanol level is then

* The apparatus is available from Messrs. Woods Bros., Glass Company Limited of Barnsley (Yorkshire) in two sizes (1 and 2 litre extraction flasks).

adjusted so that it just overflows into a setting tank (the conical flask "B"). This conical flask is filled with absolute ethanol and as the supernatant from the extraction flask overflows into it further precipitation of protein occurs. The precipitate settles readily to the bottom of this flask and does not reach the evaporation flask (flask "C") which is of 500 ml. or 1 litre capacity depending on the size of flask "A". When the 1 litre size evaporating flask is used it must be lengthened with a 3 in. adaptor to compensate for the increased size of the 2 litre extraction flask. The overflow from the settling tank refills the evaporation flask which has been previously filled to about one inch above the sidearm with 95 per cent ethanol. The rubber connectors (R1 and R2) are joined, the air leaks (L1 and L2) drawn out in a flame to fine capillaries. Suction is then applied (via T) from a water pump.

The exact pressure depends on the ambient room temperature. In England this is about 20° and the required pressure is then about 10 cm. of Hg. It is important to connect a simple manometer to the pump so that an exact pressure reading can be taken. At 10 cm. of Hg, ethanol boils at approximately 30° and, with the lower part of the evaporation flask surrounded by a water bath at 80°, the rate of boiling in the flask "C" is then rapid and controlled. The ethanol vapour condenses (condenser C1) and is returned to the extraction flask. Cycling of the ethanol is thus achieved and continuous extraction of the tissue and concentration of the extract in flask "C" established.

The pressure must not be reduced to such a degree that there is a gross loss of ethanol down the water pump. We would suggest that the pressure should be adjusted in each case by means of a capillary leak on the pump so that about a 12° temperature differential is maintained between room temperature and the evaporation temperature of the ethanol (thermometer T1).

Periodic agitation of the ethanol in flask A is accomplished by means of a screw clip on a rubber tube attached to the air leak (L1).

Extraction Times

We first demonstrated the efficiency of the apparatus by adding phenolphthalein to 100 g. of liver in the 1 litre extraction flask. This provided a simple colorimetric method of following the extraction by observing the red colour produced by adding aqueous sodium hydroxide to aliquots of ethanol taken from each of the flasks A, B and C. We found that 2 hours were necessary for complete transfer of the phenolphthalein from flask "A" to flask "C". Henceforth we used a 6 hour period for the extractions. No ethanol had to be added during this period, the apparatus worked perfectly without attention.

Compounds Investigated

Barbiturates

100 g. of liver from a case of amylobarbitone poisoning and 2 g. of tartaric acid were macerated with ethanol and extracted in the apparatus.

The contents of flasks "B" and "C" were then filtered through a Whatman No. 4 fluted filter paper and the filtrate evaporated under water pump pressure (15 mm.) to approximately 10 ml. 100 ml. of 0·5 N sulphuric acid was then added and the solution refiltered. The barbiturate was then extracted from the aqueous acidic solution with ether (200 ml.) which was washed with 20 ml. of 5 per cent aqueous sodium bicarbonate solution and then shaken twice with 20 ml. of 2·5 per cent aqueous sodium hydroxide. The alkaline aqueous extracts were acidified and re-extracted with ether. The ether extract was evaporated and the barbiturate assayed by ultra-violet spectrophotometry[2]. Comparison of the recovery using the continuous extractor with the recovery of barbiturate from the same liver using a tungstic acid method[3] showed that the extractor gave an approximately 90 per cent recovery compared with the latter method.

Alkaloids

The extraction of four alkaloids was investigated. Quinine was chosen because its high ultra-violet absorption spectrum at 347 mμ made it especially suitable for accurate spectrophotometry. Morphine was next

TABLE I

RECOVERY OF ALKALOIDS

Viscera (100 g.)	Alkaloid	Alkaloid added, mg.	Recovered, mg.	Recovery, per cent
Liver	Quinine	3·0	2·0	66
Liver	Morphine	1·5	1·2	80
Liver	Atropine	3·0	2·7	90
Brain	Cocaine	3·0	1·54	51

investigated because it is very commonly encountered and because it is an alkaloid which often presents special difficulties in extraction. Finally atropine and cocaine were added to viscera and their extraction followed. These last two compounds contain ester linkages which make them very easily hydrolysed.

The procedure for isolation was identical with that of the barbiturates described above up to and including the extraction by ether of the aqueous sulphuric acid. The aqueous phase was then made alkaline with ammonia and shaken in turn with ether and chloroform : isopropanol (5 : 1). Quinine and cocaine were estimated by ultra-violet spectrophotometry after evaporating the ether phase and dissolving aliquots of the extract in 0·1 N sulphuric acid (quinine 347 mμ) (cocaine 233 mμ) and atropine and morphine by comparative paper chromatography on aliquots of the ether and chloroform isopropanol phases respectively. The solvent system in each case was that of Curry and Powell[4] : the method of detection was Dragendorff's reagent for atropine and Marquis reagent for morphine. Paper chromatography was also used to cross check the ultra-violet spectrophotometric results for quinine and cocaine. The viscera used were liver and brain and the results are tabulated in Table I.

Glycosides

We considered that this method of extraction might be suitable for the isolation of glycosides. This is a subject which has been largely ignored in the last 20 years in toxicological literature. Digoxin and solanine were added to the contents of a stomach and small intestines and Table II shows that the results are highly encouraging.

The isolation identification and assay of these glycosides was achieved by the following procedure.

As with the barbiturates and the alkaloids, the ethanol in the extraction flask was evaporated under water pump pressure to about 10 ml. Water (100 ml.) and dilute sulphuric acid (10 ml.) were added and the turbid solution was filtered. After adjusting to pH 5 digoxin was extracted from the aqueous solution by two extractions with 100 ml. of chloroform which was separated off and evaporated. Solanine was then extracted with warm pentanol after making the aqueous phase slightly alkaline with ammonia. This alcohol was also evaporated under reduced pressure.

TABLE II

RECOVERY OF GLYCOSIDES

Viscera	Glycoside	Added, mg.	Recovered, mg.	Recovery, per cent
Stomach and small intestine contents	Digoxin	7·5	5·5	73
Liver (100 g.)	Digoxin	2·5	2·0	80
Stomach and small intestine contents	Solanine	10·0	4·0	40

Proof of identity and quantitative assay were achieved as follows. The extract containing digoxin was dissolved in 70 per cent aqueous ethanol and aliquots were examined by paper chromatography in the following solvent systems[5,6]:

(*a*) chloroform : methanol : water (10 : 2 : 5) (organic layer used);

(*b*) chloroform : benzene : water (65 : 35 : 50) + 7·5 ml. methanol (organic layer used).

The first spray was 5 per cent *m*-dinitrobenzene in benzene. After heating in the oven at 80° for 15 minutes the paper was dipped in 20 per cent sodium hydroxide solution. Digoxin showed as a blue spot at R_F 0·71 in solvent (*a*) and R_F 0·32 in solvent (*b*). Two other methods of detection were sprays of 5 per cent trichloracetic acid and 20 per cent antimony trichloride in dry, alcohol free, chloroform; in each case the paper was heated at 80° for 3 minutes after spraying. We felt that further proof of identity might be desirable and so investigated the chromatography of digoxigenin. We found this aglycone gave a green fluorescence under ultra-violet light on the solvent front (solvent system (*a*)) when the paper was sprayed with the antimony trichloride reagent and heated at 70° for 3 to 5 minutes. We demonstrated the conversion of the recovered digoxin to digoxigenin by warming with dilute hydrochloric acid at 100° for 30 minutes. We also showed that the apparatus and method outlined above gave excellent recovery of digoxin added to liver but that when digoxigenin

was added to liver it could not be recovered. We have as yet no explanation for this finding.

Solanine was identified and estimated by a comparative chromatogram using Curry and Powell's system[4] and Clarke's reagent[7] (1 per cent paraformaldehyde in concentrated phosphoric acid) poured on to the paper. The solanine was recovered unchanged; there had been no loss of the sugars to give solanidine.

Discussion

An apparatus has been developed for the extraction of many classes of poisons from viscera. Ethanol was chosen not only because it is a good solvent for the poisons, but because in many countries the viscera are submitted to the laboratory already preserved in it.

The efficiency of the extraction has been followed using the barbiturates, alkaloids and glycosides. Substances were chosen which were either insoluble in the usual solvent systems and did not fit in the general extraction scheme or were substances whose instability was such that their extraction was particularly difficult.

In all forensic work speed is often of vital importance and we suggest that this extractor will materially reduce not only the time required but also the volume of ethanol necessary for the extraction of many poisons.

In the examples discussed above, we deliberately added milligram quantities of poison to the viscera: however, the detection and assay were always accomplished with less than 1 per cent of the extract and the scheme should be applicable in cases of poisoning by these classes of poison.

It seems probable that such an apparatus might be of use in many other branches of chemistry.

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PHARMACOPOEIAS AND FORMULARIES

BRITISH VETERINARY CODEX SUPPLEMENT 1959*

REVIEWED BY ALASTAIR N. WORDEN

"Having given careful consideration to the scope of the proposed new edition of the Codex, the British Veterinary Codex Committee decided that, before starting on a complete revision, the needs of users of the Codex would best be served by first publishing a supplement to the present edition." The publication of the *British Pharmacopoeia of 1958* and of the *British Pharmaceutical Codex of 1959* was awaited in order to embrace the changes likely to be made in the new editions of these two works, and thanks to liaison with the British Pharmaceutical Codex Revision Committee it has been possible to publish the B. Vet. C. Supplement almost simultaneously with the new B.P.C.

The supplement contains 47 additional, and 38 amendments to existing, Part I Monographs; 6 additional, and 42 amendments to existing, Part III Monographs. Of the 47 additions to Part I, however, 33 relate to new substances in the B.P. 1958 or the B.P.C. 1959.

In Part I, special interest attaches to the anthelmintics, Bephenium, Picadex and to the four salts of Piperazine, to the insecticide Dieldrin, to the synthetic steroids, Prednisolone and Prednisone and their Acetates, and to substances such as glucogenic agents (Propylene Glycol and Sodium Propionate), Calcined Magnesite, Acinitrazole, Furazolidone and Nicarbazin—all of which are likely to have wide usage in animal production in circumstances which rarely permit of full veterinary control. There are indeed important differences between medical and veterinary usage of potent materials, especially in the case of the so-called economic animals, and this underlines the need for standards such as are laid down in the B. Vet. C., and for widespread compliance with them. In view of the fact that a standard has now been laid down for an oral magnesium preparation which ". . . consists chiefly of magnesium oxide, and is usually prepared by igniting naturally occurring magnesite," it may be questioned whether in fact the standard should be expressed in terms of the oxide and not of elemental magnesium. It would be unfortunate if there arose the same confusion over magnesium as has arisen earlier in agricultural circles from the use of "CaO" and "P_2O_5".

In Part II, new monographs have been added on Canine Distemper and Hepatitis Antiserum, Johne's Disease Vaccine (Living), Myxomatosis Vaccine (Living), and Ovine Enzootic Abortion Vaccine, Attenuated (Living), and there have been some important amendments to existing monographs. Usage of some of these materials by lay persons depends upon other circumstances, but the fact that certain sheep vaccines and sera are widely employed by farmers themselves adds to, rather than detracts from, the need for stringent standards.

In Part III chief interest centres upon a monograph on Antibiotics as Dietary Supplements. The very large sales of antibiotic supplements, the use for certain purposes or on certain premises of much higher levels than those normally recommended, the debate that continues upon the disadvantages and dangers of permitting the development of resistant strains of micro-organisms in stock—and in their human attendants—are all factors that emphasize the need for adequate control of this category of material.

* Published by direction of the Council of the Pharmaceutical Society of Great Britain. Pp. xviii + 134 (including Index). The Pharmaceutical Press, 17, Bloomsbury Square, London, W.C.1. 35s. (Postage, U.K. 1s. 3d.; Overseas, 2s.).

The six years that have elapsed since the original publication of the B. Vet. C. have seen an important and useful rôle established for it. The veterinary field is developing rapidly and it will surely not be long before a new edition, or further supplements, appear.

BOOK REVIEWS

AN INTRODUCTION TO PHARMACOLOGY. By J. J. Lewis. Pp. xii + 826 (including Index). E. and S. Livingstone Ltd., Edinburgh, 1960. 55s.

Need has been felt for a longer textbook of pharmacology, dealing with all the drugs in use in clinical practice and at a price within the means of the undergraduate student. The author has attempted to meet that need in this book which is a careful and comprehensive summary of the chemical structures, the effects and the main clinical uses of drugs. The care with which this book has been written and prepared is obvious; there are exceedingly few misprints. The absence of references which might act as an introduction to the literature, the confinement of the section on quantitative and general pharmacology to one brief introductory chapter, and the emphasis on the overall effects of drugs in preference to mode of action makes this a book much less suitable for the student of science who is reading pharmacology than for the student of medicine.

M. F. LOCKETT.

HALE WHITE'S MATERIA MEDICA, PHARMACOLOGY AND THERAPEUTICS. 31st Edition. By A. H. Douthwaite. Pp. viii + 525 (including Index). J. and A. Churchill, Ltd., London, 1959, 25s.

Knowledge is advancing increasingly rapidly in all branches of medicine. It is probably going to advance more quickly in the future. The knowledge we have already will be superseded in many respects. Consequently, it becomes increasingly difficult to decide what factual material should be put before medical students. The less the better is a maxim which will comfort both the overloaded student and the harassed teacher, and it has the merit that the less purely factual matter is taught, the more time is left to consider the evidence for the facts and the proper weighing of evidence. A student who has learnt how to assess evidence will be an intelligent doctor ten years hence. One who has not will be a mixed-up victim of superseded knowledge and commercial pressure, and his patients will suffer accordingly.

Hale White's Materia Medica is an old book which has maintained its popularity to the extent of thirty-one editions since it first appeared in 1892. The newest edition is up to date in the sense that it deals with drugs such as chlorothiazide and dexamethasone, and traditional in that it preserves its authoritarian approach, giving no evidence for the statements made and no references to places where the evidence can be found. Characteristically, there is no entry in the index for "therapeutic trial", "clinical trial" or "trial" and it does not appear that this crucial subject is discussed at all. Nor is there any reference to the effects of dummy medication in health and disease, in spite of their fundamental importance in evaluating therapeutic efficiency. It does not appear, in fact, that the statements in the book are based on scientific evidence at all, and it does not encourage the search for such evidence. This book is not therefore to be recommended.

M. WEATHERALL.

METHODEN DER ORGANISCHEN CHEMIE (Houben-Weyl). Fourth Edition. Edited by Eugen Müller. Volume 1, Part 2. Allgemeine Laboratoriumspraxis. Pp. xlviii + 1017 (including Index and 680 illustrations). George Thieme Verlag, Stuttgart, 1959. Moleskin, DM 196.00.

The second part of volume I, like its counterpart which appeared some 18 months ago, is devoted to the technique of general laboratory practice. Although it contains much of general interest to all laboratory workers, it will be of special interest to those engaged in pharmaceutical work. The first few chapters present an excellent summary of the problems of comminution; sifting of solids; the mixing of gases, liquids, powders and plastics; emulsions, emulsifying agents; foams and antifoaming agents. The remainder of the volume covers a range of practical techniques for the preparation, purification, handling and storage of materials with special reference to problems of physical state. The subject matter ranges over methods of working with gas streams the use of condensed gases (ammonia, sulphur dioxide and hydrofluoric acid); explosives; small-scale methods; working under anaerobic or anhydrous conditions, under pressure and under vacuum; the measurement, recording and regulation of temperature; drying agents and the purification of organic solvents The chapter on laboratory accidents and the danger of carcinogenesis completes an extremely useful manual, and provides a useful reminder of the care which is always necessary in laboratory work. The volume is excellently referenced, up to date and beautifully presented in the same excellent style of its predecessors in the series.

J. B. STENLAKE.

LETTERS TO THE EDITOR

A Simple Device for Testing the Spreadability of Pharmaceutical Suspensions for External Use

SIR,—In the course of my investigations into various aspects of pharmaceutical suspensions with emphasis upon the application of statistical methods[1,2], a simple device called a grindometer generally used to evaluate the fineness of printing inks[3,4] proved after careful experimentation and a statistical treatment of experimental data, to be useful when measuring the spreadability of pharmaceutical suspensions destined for external use. From my experience, the underlying principle of the grindometer, the testing of dispersibility, can also be applied to powdered medicinal substances.

The grindometer used in my work consists of a mild steel block; in the upper surface there is machined an incline, the bottom and sides of which are highly

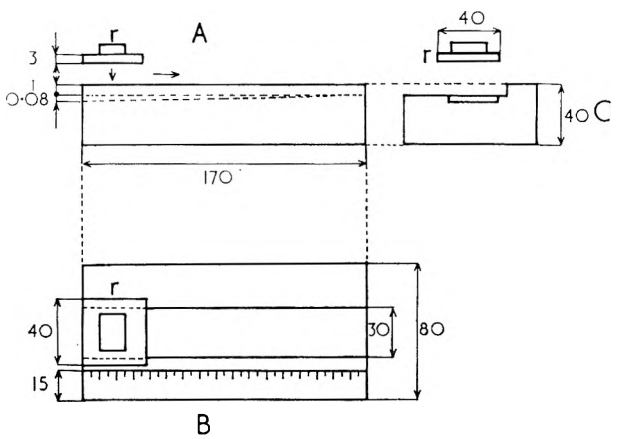

FIG. 1. Schematic sketch of the grindometer. A = front view; B = top view; C = side view; r = rubbing spatula. All dimensions are in millimetres.

polished. An empirical scale projects above the upper surface and is marked along the incline. A separate part of the device is a rubbing spatula. The dimensions are indicated in Figure 1.

In use a sample of 0·2 ml. of the suspension is placed on the incline at the lower end of the grindometer. After 10 seconds the rubbing spatula is placed on the sample and after a further 10 seconds it is moved without applying pressure to it; a smooth linear movement is achieved by resting the rubbing spatula against the projection of the scale during the movement. The value on the scale up to which the coherent layer of the sample then extends is recorded.

Preliminary results have been obtained on the influence of Tween 80 on the spreadability of a test composition. Tween 80, 1 per cent, was added to a suspension the composition of which was as follows: zinc oxide 15 per cent, talc 15 per cent, glycerol 10 per cent, Adulsion ST "Kalle" ("a form of methyl cellulose and carboxymethyl cellulose") 2 per cent, and water 57 per cent. Since it was expected that the addition of Tween 80 would cause an improvement in spreadability and since each experimental value was known before the next experiment was carried out, the results were treated by the pertinent method of sequential analysis[5,6].

As far as the mode of sequential analysis which I have used is concerned, the following data were known or fixed before the start of the actual experiments testing the influence of the addition of Tween 80 to the above-mentioned suspension: mean value of spreadability of the suspension without Tween 80 = 4·7; difference in spreadabilities of suspensions with and without Tween 80 which it is important to detect = 1·0; standard deviation = 0·6; risk of asserting a

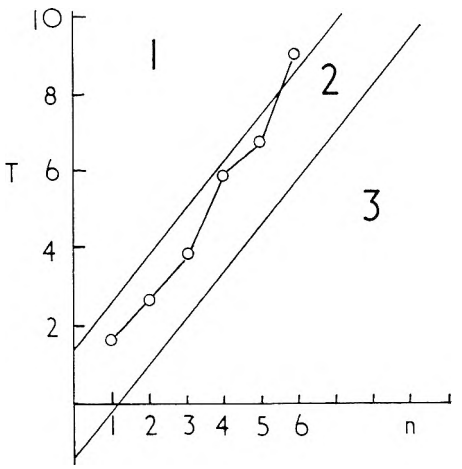

FIG. 2. Sequential test for measuring the improvement in spreadability caused by the addition of 1 per cent Tween 80 to a suspension. n = number of experiments; T = cumulative total of coded values (coded value = actual value − 4·0) of spreadability. Zone 1 = real improvement in spreadability; zone 2 = the experiments to be continued; zone 3 = no improvement in spreadability.

significant difference when none exists = 0·02; risk of failing to detect a significant difference = 0·02. These values served as fundamentals for constructing a graph (Fig. 2) which illustrated the relation between the cumulative value of spreadability of the suspension with Tween 80 (T) and the number of experiments (n). The graph was divided into three zones each of which indicates a proper decision to be taken when T is in or enters this zone: zone 1: there is a real improvement in spreadability caused by the addition of Tween 80; zone 2: the experiments are to be continued since the available data are insufficient; zone 3: there is no significant difference in spreadabilities of the suspensions with and without Tween 80. Thus the testing was to be terminated when T entered zone 1 or zone 3.

As is obvious from Figure 2, the decision that the spreadability of the suspension with Tween 80 is better than of that without Tween 80 was taken after the sixth experiment.

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May 6, 1960.

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The Critical Micelle Concentration of Polyethyleneglycolmonocetylether

SIR,—The recent paper of Elworthy[1] reporting the determination of the critical micelle concentration (CMC) of a commercial sample of cetomacrogol of molecular weight 1210 has prompted us to report values we have obtained for two commercial samples of polyethyleneglycolmonocetylether; one designated A complied with the B.P.C. 1959 requirements for Cetomacrogol 1000 and the other designated B failed to comply by virtue of 0·1 per cent excess

TABLE I

VALUES FOR THE CRITICAL MICELLE CONCENTRATION OF POLYETHYLENEGLYCOLMONOCETYLETHERS

Method of determination		Critical concentration per cent	
		Batch A	Batch B
Surface tension	1	0·00135	0·00082
	2	0·00119	0·00105
Light absorption ..	1	0·00132	0·00085
	2	0·00145	0·00093

water. In our experiments CMC values were obtained by observing changes in surface tension with concentration as measured by the Du Nouy tensiometer. Determinations were also made with the iodine method of Ross and Oliver[2], using a Unicam SP500 spectrophotometer. Our results are summarised in Table I.

Carless and Nixon[3] report values of 10^{-6} to 10^{-7} M with the Du Nouy tensiometer for the CMC of a sample of cetomacrogol which according to their data contained more ethylene oxide residues than that required by the B.P.C. 1954. As these workers assumed a molecular weight of 1300, their values for the CMC may be expressed as 0·00013 to 0·000013 per cent.

Working with specially prepared samples of polyoxyethylene alcohols, Cohen[4] concluded from surface tension measurements, that for a given alcohol, the CMC was independent of the number of ethylene oxide units. This is to be contrasted with the findings of Becher[5], whose work indicates that for a given alcohol, the CMC varies as the number of ethylene oxide units is increased. The value for the CMC obtained by Cohen[4] for the reaction products of ethylene oxide with cetyl alcohol, was $\frac{M}{50,000}$, which for $C_{16}H_{33}O[CH_2CH_2O]_{20}H$, gives a value of 0·00225 per cent.

We have, therefore, values of 0·007[1], 0·001–0·0009 and 0·0001 to 0·00001[3] per cent for the CMC of commercial samples and 0·00225 per cent for a pure sample of this class of compound.

Kushner and Hubbard[6] suggested that variable composition of Triton x-100, an alkylaryl polyethylene oxide, was the cause of their failure to reproduce the value of the CMC reported by Gonick and McBain[7], and it is interesting to speculate that a similar explanation is applicable to the variable results for commercial samples of polyethyleneglycolmonocetylethers.

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May 10, 1960.

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