RESEARCH PAPERS

THE EFFECT OF MUSCLE CONTRACTION ON THE BLOOD FLOW AND ON THE VASCULAR RESPONSES TO ADRENALINE, NORADRENALINE AND ISOPRENALINE IN INDIVIDUAL SKELETAL MUSCLES OF THE CAT

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The effect of muscle contraction on the rate of venous outflow and on the vascular responses to adrenaline, noradrenaline and isoprenaline was studied in individual muscles of the hind limbs of cats under chloralose anaesthesia. The maximum hyperaemia arising from contraction was produced when maximal twitches were elicited at frequencies which varied in different experiments from 1 to 8 per second, that is, frequencies well below those required to produce tetanus. During prolonged tetanic contractions the tension in the muscles soon fell to become constant at a lower level. This fall in tension reduced the mechanical impedance to flow and allowed vasodilatation to occur. In the tibialis anterior muscle the maximum hyperaemia was reached during the constant tension phase of the tetanus but in the soleus, the tension of which is maintained at a higher level, mechanical compression impeded the flow throughout the contraction. Both local vasodilator and vasoconstrictor actions of the sympathomimetic amines were reduced during the hyperaemia of muscle contraction. During contraction the muscle blood flow was markedly influenced by changes in mean arterial pressure. Thus isoprenaline, although a dilator substance in resting muscle, often passively reduced the blood flow through a contracting muscle, when injected intravenously, because of its hypotensive action. The reverse occurred with intravenously administered adrenaline and noradrenaline. The rise in blood pressure produced by these amines often forced more blood through the contracting muscle despite a weak local constrictor action.

THERE are a number of examples of published work in which conclusions concerning the effects of drugs on skeletal muscle contractions have been based on a preconceived knowledge of the vascular responses to the drugs in resting muscle. Particularly is this so in studies of the effects of sympathomimetic amines on the contractions of skeletal muscle. The effect of a drug on the blood flow through a contracting muscle may, however, be different from that in a resting muscle. For example, it has been shown that the local vasoconstrictor effect of adrenaline may be reduced[1–4], or abolished[5] during the hyperaemia accompanying a sustained tetanus. It was, therefore, considered of interest to study the effects of muscle contraction on the local vascular responses to other sympathomimetic amines as well as to adrenaline. Noradrenaline and isoprenaline were chosen for study because they are known to possess opposite actions on the blood flow through resting muscles[6–11]. In addition, a comparison was made of the effects of muscle contraction itself on the circulation through a slow (soleus) and a fast (tibialis anterior) contracting muscle.

The results emphasise the possible fallacies that may arise in drawing conclusions in the absence of a concomitant blood flow recording for during the vasodilatation accompanying muscle contraction, the local vascular actions of intravenously administered drugs may be completely masked by passive changes in flow resulting from alterations in mean arterial pressure.

Methods

Cats were anaesthetised with chloralose only (80 mg./kg.) injected into the subcutaneous vein of the fore-limb. The preparation for the simultaneous recording of the contractions of and the venous outflow from the tibialis anterior, the gastrocnemius-plantaris or the soleus muscle was as previously described[9,12]. Shielded silver electrodes were placed on the sciatic nerve which was tightly ligated central to the electrodes. Contractions of the muscle were excited by rectangular pulses of 0·2 msec. duration and of twice the voltage necessary to evoke a maximal twitch. Blood pressure was recorded from a carotid artery, a blood pressure stabiliser[13,14] being connected when required. Drugs were injected intravenously through a cannula in a jugular vein or intra-arterially from a micro-syringe through a cannula in the cut central end of a branch of the femoral artery (for further details see Bowman[9]). The drugs used were (−)-adrenaline bitartrate, (−)-noradrenaline bitartrate and isoprenaline sulphate. The doses quoted refer to the quantity calculated as base. Solutions were prepared in 0·9 per cent w/v NaCl solution.

Results

Preliminary experiments showed that stimulation of the sciatic nerve with the voltages and frequencies used in these experiments was without effect on the blood flow through muscles paralysed by decamethonium. It was concluded, therefore, that concomitant stimulation of sympathetic nerves in the sciatic trunk did not influence the results to be described.

The Effect of Contraction on Venous Outflow

A series of maximal twitches, either of the tibialis anterior or of the soleus, produced an increase in the venous outflow from the contracting muscle. This effect was evident with rates of stimulation as low as once every 10 seconds (Fig. 1). The rate of flow at first increased as the frequency of stimulation was increased and a linear relation existed between the two until the flow reached a maximum at frequencies which varied from 1 to 8 per second in different experiments, but which were usually in the range of 2 to 4 per second. The increase in flow began immediately the rate of stimulation was speeded and usually reached a steady level within 30 seconds. The return of the flow to a slower, steady rate on reducing the frequency was more gradual, often taking as long as 8 to 10 minutes.

A few instances were recorded where the vessels of the resting muscle temporarily acquired a state of rhythmical activity. That is, they began to dilate and constrict alternately. McDowall[15] also experimenting on

cats, recorded a similar response. The rhythm was abolished during the hyperaemia which ensued when the muscles were made to contract, but often recurred on stopping the stimulation. Figure 2 shows rhythm of this type in the muscle vessels which was not accompanied by corresponding changes in mean arterial pressure.

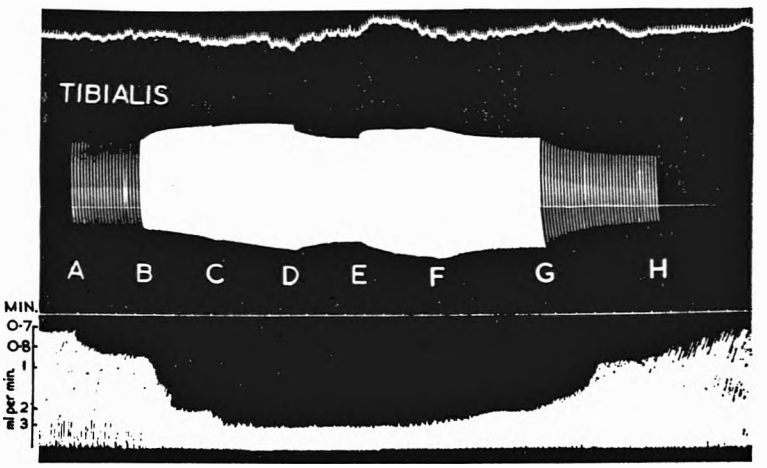

FIG. 1. Cat, 3·4 kg. The effect of a series of maximal twitches on the venous outflow from the tibialis anterior muscle. Upper record:—blood pressure; middle record:—indirectly excited maximal twitches; lower record:—venous outflow. Stimulation frequencies:—Between A and B and G and H, once every 10 sec.; between B and C and F and G, once per sec.; between C and D and E and F, twice per sec.; between D and E, four times per sec.

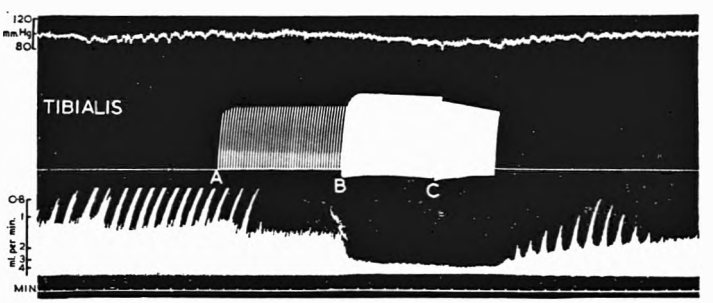

FIG. 2. Cat, 4 kg. Rhythmical activity of the blood vessels. Maximal twitches elicited, between A and B, once very 10 sec., between B and C, once per sec. and beginning at C, twice per sec.

Changes in venous outflow during tetanic contractions depended on the muscle under study, the duration of the tetanus and the tension produced. There was always an initial short-lasting increase in venous outflow as the muscle first contracted (Figs. 3B, 4, 5, 6). This was followed by a decrease in flow which lasted as long as the muscle tension

remained at its maximum. The extent of the decrease in flow depended on the magnitude of the tension produced (Fig. 3A). In these experiments the muscles were unable to maintain the initial tension which soon relaxed to a lower level where it was maintained for several minutes.

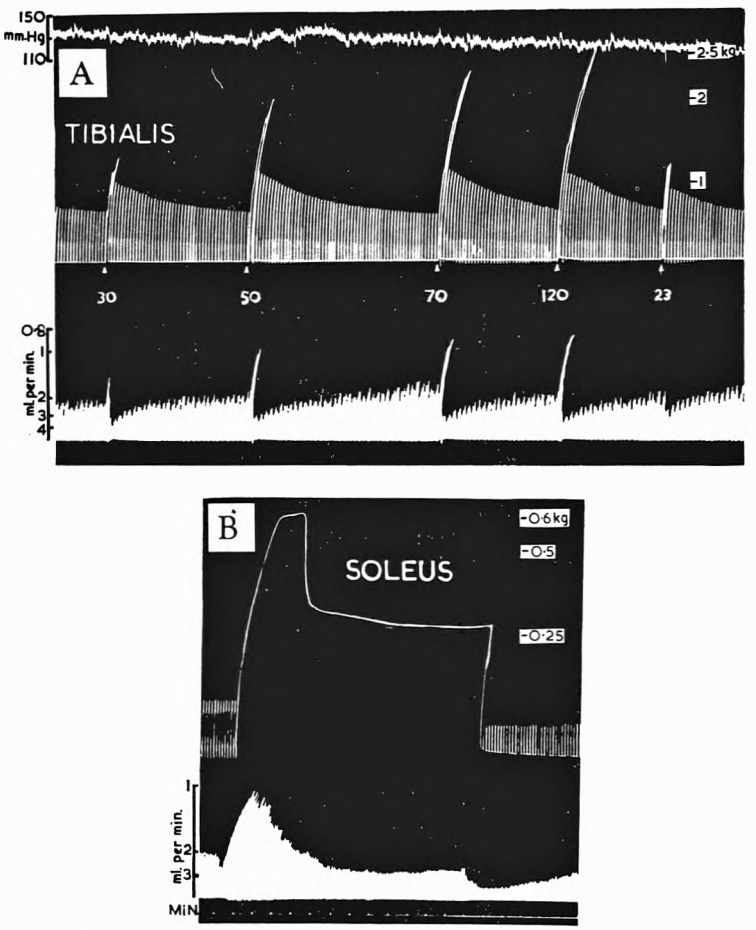

FIG. 3. The effect of tetanic contractions on venous outflow.
A.—cat, 3·6 kg. Maximal twitches elicited once every 10 sec. and tetani of 3 sec. duration interpolated. The figures below the middle record show the frequency of tetanic stimulation per sec.
B.—cat, 3·9 kg. Maximal tetanus of soleus muscle of 12½ min. duration elicited by stimulation at a frequency of 70 per sec.

As the tension fell, the blood flow increased until it reached a constant level at the same time as the tension reached its constant level (Figs. 3B to 6). The initial tension was maintained longer by the soleus at all rates of stimulation than it was by the tibialis anterior muscle. Maximum tetanic tension of the soleus muscle was produced by rates of stimulation of 50 to 75 per second and the initial tension was maintained for 30 to 90

seconds. Maximum tetanic tension of the tibialis anterior muscle was produced by rates of stimulation of 100 to 120 per second and the initial tension was maintained for 15 to 20 seconds. The tension of the tibialis anterior in a maximum tetanus usually became constant at $\frac{1}{4}$ to $\frac{1}{3}$ of the initial tension, whereas the tension of the soleus in a maximum tetanus became constant at $\frac{1}{2}$ to $\frac{2}{3}$ of the initial tension. The hyperaemia which

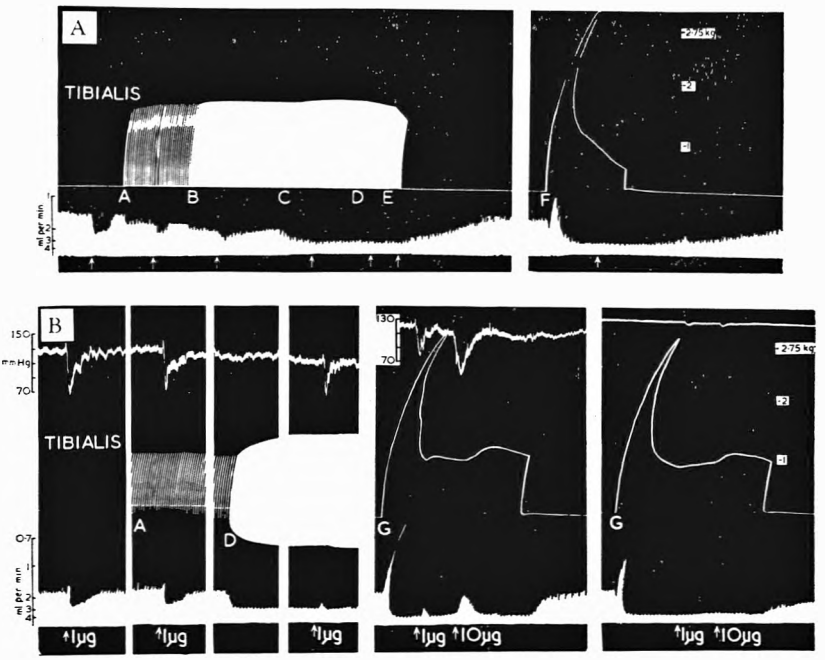

FIG. 4. The effect of muscle contraction on the local vasodilatation produced by isoprenaline.
A.—cat, 3·8 kg. At the arrows, 0·4 μg. isoprenaline administered intra-arterially. Stimulation frequencies:—between A and B, once every 10 sec.; between B and C, once every 5 sec.; between C and D, once every 2 sec.; between D and E, once per sec. Beginning at E, twice per sec. and at F, 50 per sec.
B.—cat, 4·2 kg. At the arrows isoprenaline administered intravenously. Stimulation frequencies:—between A and D, once every 10 sec.; beginning at D, once per sec. and at G, 120 per sec. The record on the extreme right shows the effect of isoprenaline after stabilising the blood pressure.

occurred during the constant tension phase of the tetanus was relatively greater in the tibialis anterior than in the soleus muscle. However, a further increase in flow from the soleus muscle occurred after the tetanus (Fig. 3B) whereas the hyperaemia reached its maximum during tetanus of the tibialis anterior and did not increase afterwards (Figs. 4A, 5, 6). The maximum rate of blood flow during a tetanus of the tibialis anterior, or immediately after a tetanus of the soleus muscle, did not exceed the greatest hyperaemia produced by a rapid series of single twitches, but was usually of equal value (Figs. 4A, 5, 6).

The Effect of Muscle Contraction on the Blood Flow Responses to Adrenaline, Noradrenaline and Isoprenaline

In the resting muscle, adrenaline and noradrenaline may produce an increase in flow, a decrease in flow or a compound response depending

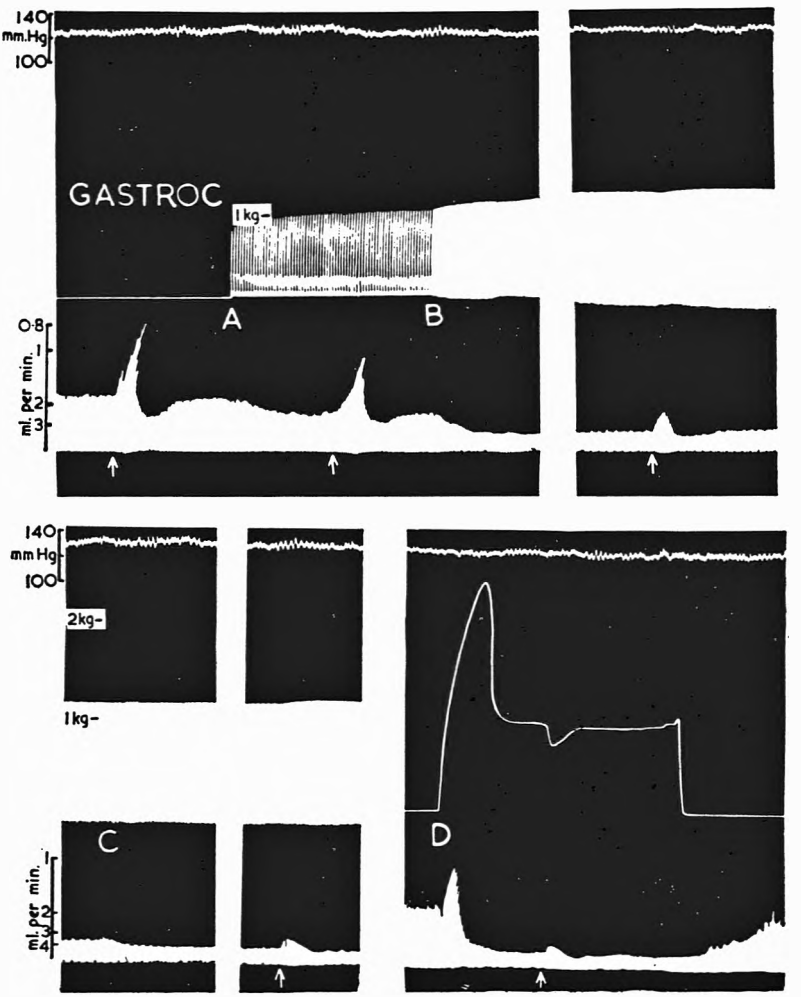

FIG. 5. Cat, 3·4 kg. The effect of muscle contraction on the local vasoconstriction produced by intra-arterially administered adrenaline. At the arrows, 3 μg. adrenaline injected intra-arterially. Stimulation frequencies:—between A and B, once every 10 sec.; between B and C, once every 3 sec.; beginning at C, once every 1·5 sec. and at D, 50 per sec.

on the dose administered, the route of injection, the general arterial blood pressure and the vasomotor tone in the muscle[9]. Isoprenaline always causes vasodilatation[11].

The increase per cent in the rate of blood flow produced by isoprenaline

or by dilator doses of adrenaline was reduced during the hyperaemia accompanying muscle contraction. The greater the rate of stimulation, the greater the hyperaemia and the greater the reduction in the effect of these amines. When the maximum vasodilatation arising from muscle contraction had been obtained it was impossible to produce any further dilatation with intra-arterially administered isoprenaline (0·002 to 0·4 μg.) or adrenaline (0·001 to 0·05 μg.), both amines then being completely without effect on the blood flow. Figure 4A illustrates the effect of increasing the frequency of stimulation on the dilator response to intra-arterially administered isoprenaline. When the muscle vessels were at, or approaching the stage of maximum dilatation arising from muscle

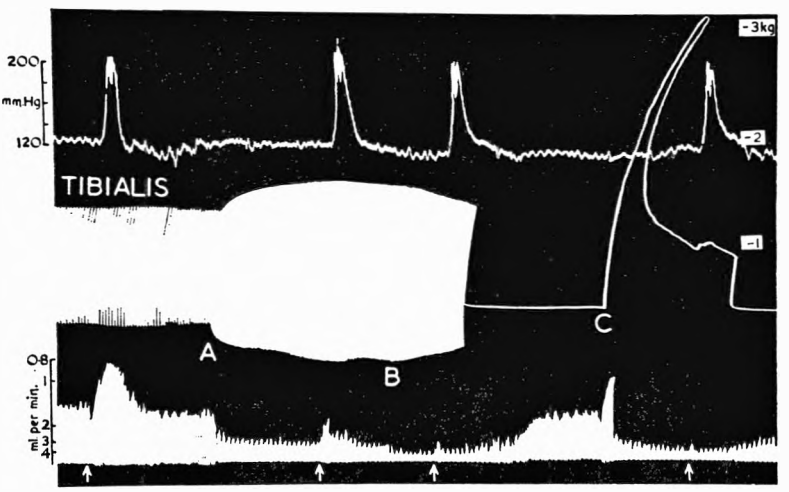

FIG. 6. Cat, 3·9 kg. The effect of muscle contraction on the local vasoconstriction produced by intravenously administered noradrenaline. At the arrows, 10 μg. noradrenaline injected intravenously. Stimulation frequencies:—initially once every 10 sec.; between A and B, once per sec.; beginning at B, twice per sec., and at C, 50 per sec.

contraction, the intravenous administration of isoprenaline (0·25 to 2·5 μg./kg.) usually caused a marked reduction in venous outflow from the muscle. The intravenous administration of dilator doses of adrenaline (0·25 to 2·5 μg./kg.), on the other hand, still caused some increase in the rate of blood flow. The use of the blood pressure stabiliser showed that both of these responses were passive effects caused by the changes in mean arterial pressure. Figure 4B illustrates the effects of intravenously administered isoprenaline.

Vasoconstriction produced by adrenaline or noradrenaline (1 to 3 μg. i.a. or 2·5 to 5 μg./kg. i.v.) was reduced during the hyperaemia of muscle contraction. The greater the rate of stimulation, the smaller the vasoconstriction became. Even when maximum dilatation arising from contraction had been produced, further increase in the frequency of stimulation caused a further reduction in the vasoconstrictor action of adrenaline

and noradrenaline. Figures 5 and 6 illustrate these effects with intra-arterially administered adrenaline and intravenously administered noradrenaline respectively. The intravenous administration of small doses of adrenaline and noradrenaline which caused a weak vasoconstriction in the resting muscles, frequently caused only an increase in blood flow during the hyperaemia of contraction. When, by means of the blood pressure stabiliser, the blood pressure was prevented from rising, the same doses were either without effect or again caused a weak vasoconstriction in the contracting muscles.

Discussion

It has long been established that muscle contraction is accompanied by vasodilatation[1,2,16 to 25], and the present experiments show that in the experimental animal anaesthetised with chloralose the maximum effect occurs at stimulation frequencies well below those required to produce tetanus. During tetanic contractions there is an initial sharp increase in venous outflow as the muscle contracts. No initial increase in flow occurred, however, in some earlier experiments in which the femoral artery flow was recorded and in common with others[2,26], it is concluded that this effect is simply due to mechanical compression forcing out blood from the muscle. Subsequent to the initial increase in venous outflow, a short period occurs during which the flow from the muscle is decreased. This is considered by many workers[2,16,17,21 to 28] to be due to the mechanical compression of the muscle vessels impeding the flow and the present results support this view, since the extent and duration of the decrease in flow proved to be dependent on the extent and duration of the initial high tension in the muscle. During prolonged tetanic stimulation in the experimental animal, the initial tension in the muscle soon falls, probably at least partly as a consequence of the reduced blood flow. It then becomes constant at a lower level. During the fall in tension, the venous outflow increases to a level 2 to 6 times that of the flow from the resting muscle. It appears, therefore, that vasodilatation is made possible by the fall in tension and the consequent reduction in mechanical compression. Kramer and Quensel[21] were of the opinion that 5 seconds was the longest duration of the mechanical impedance to flow. But, in the present experiments this impedance lasted as long as the initial tension was maintained, which in turn depended on the muscle under study and the frequency of stimulation. Since different muscles maintain the initial tension for different periods, vascular changes can accurately be related to tension changes, only when, as in the present experiments, the blood flow from individual muscles is recorded.

A voluntary muscular contraction is the algebraic sum of the contractions and relaxations of the large number of motor units, each of which is contracting irregularly, asynchronously and intermittently. In an experimental electrically produced tetanus, on the other hand, all the muscle fibres involved are in sustained synchronous contraction. Since the blood flow changes during a tetanus are influenced by the tension in the muscles, the results obtained during experimental electrically produced

tetani cannot be truly related to the changes which occur in voluntary muscular movements. Presumably, during voluntary contractions, there will be vasodilatation in those motor units which are relaxed and mechanical compression in those which are contracted. The overall changes in the rate of blood flow will not, therefore, be so marked as those seen in the experimental animal.

The results obtained with sympathomimetic amines emphasise the fact that it is impossible accurately to predict the effect of a drug on the blood flow through a contracting muscle, from a knowledge of its action in resting muscle. As might be expected, the effects of vasodilator doses of the drugs are relatively smaller when the vessels are already widely dilated due to contraction. The vasoconstrictor actions of adrenaline and noradrenaline are also reduced during muscle activity. This result with adrenaline confirms that of other workers[1 to 5].

During contraction the muscle blood flow is markedly influenced by changes in mean arterial pressure. Since both local dilator and constrictor actions of the drugs are reduced during muscle activity, the changes in blood flow after intravenous administration will be mainly a consequence of the effect on the general arterial blood pressure. Thus isoprenaline, although a dilator substance in resting muscles, may passively reduce the flow through contracting muscles because of the fall in blood pressure produced. The reverse is true with adrenaline and noradrenaline. The rise in blood pressure produced by these amines may force more blood through the contracting muscles because of the weakened local constrictor action.

References

1. Rein, *Klin. Wschr.*, 1930, **9**, 1485.
2. Bülbring and Burn, *J. Physiol.*, 1939, **95**, 203.
3. Rein and Schneider, *Z. Biol.*, 1931, **91**, 13.
4. Wakim and Essex, *Circulation*, 1952, **5**, 370.
5. Mertens, Rein and Valdecassas, *Pflüg. Arch. ges. Physiol.*, 1936, **237**, 454.
6. Folkow, Frost and Uvnäs, *Acta physiol. scand.*, 1948, **15**, 412.
7. Cobbold and Vass, *J. Physiol.*, 1953, **120**, 105.
8. Dörner, *Arch. exp. Path. Pharmak.*, 1954, **221**, 286.
9. Bowman, *J. Pharm. Pharmacol.*, 1959, **11**, 104.
10. Chen, Portman, Russell and Ensor, *J. Amer. pharm. Ass., Sci. Ed.*, 1951, **40**, 273.
11. Bowman, *J. Pharm. Pharmacol.*, 1959, **11**, 143.
12. Bowman and Zaimis, *J. Physiol.*, 1958, **144**, 92
13. Krayer and Verney, *Arch. exp. Path. Pharmak.*, 1936, **180**, 75.
14. Ginzel and Kottegoda, *J. Physiol.*, 1954, **123**, 277.
15. McDowall, *ibid.*, 1950, **111**, 1.
16. Gaskell, *J. Anat. Lond.*, 1876–77, **11**, 360.
17. Gaskell, *ibid.*, 1876–77, **11**, 720.
18. Verzar, *J. Physiol.*, 1912, **44**, 243.
19. Keller, Loeser and Rein, *Z. Biol.*, 1930, **90**, 260.
20. Rein, Mertens and Schneider, *Pflüg. Arch. ges. Physiol.*, 1935, **236**, 636.
21. Kramer and Quensel, *ibid.*, 1937, **239**, 620.
22. Grant, *Clin. Sci.*, 1938, **3**, 157.
23. Barcroft and Millen, *J. Physiol.*, 1939, **97**, 17.
24. Barcroft and Dornhurst, *ibid.*, 1949, **109**, 402.
25. Hilton, *ibid.*, 1953, **120**, 230.
26. Anrep, Blalock and Samaan, *Proc. roy. Soc. B.*, 1933, **114**, 223.
27. Dolgin and Lehman, *Arbeitsphysiologie*, 1930, **2**, 248.
28. Anrep and von Saalfield, *J. Physiol.*, 1935, **85**, 375.

COMPARATIVE EFFICACY OF BACTERICIDAL COMPOUNDS IN BUFFER SOLUTIONS*

PART I

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A standardised membrane filter technique, adapted to the counting of organisms previously exposed to disinfectants, has been compared statistically with the pour-plate method. The membrane filters were found to be as suitable as pour-plates for counting viable organisms. The filter method is superior in disinfection experiments, since any quantity of test-mixture can be filtered and the danger of bacteriostasis is excluded. In disinfection experiments with high mortality levels, a great increse in variation occurred in replicate tests. Enriched media were not found to be superior to ordinary nutrient agar for the incubation of filter discs. The Gram-positive organisms tested showed a marked decrease in viable cells after storage at room temperature in slightly acid phosphate solutions for 24 hours, whereas in neutral and slightly alkaline solutions the viable count remained constant. The Gram-negative test organisms were not significantly affected by the phosphate buffer solutions.

THE object of this study was to compare the bactericidal activity of a number of disinfectants suitable for pharmaceutical solutions. A review of the literature has shown that there is little information available about the effect of the pH on the bactericidal activity of these compounds; for this reason, tests were made at four pH values from 4 to 8·5. The cell density chosen for the tests was such that the disinfectants were able, in concentrations used in pharmaceutical solutions, to sterilise the suspensions within periods varying between 30 minutes and 24 hours.

CHEMICAL

With three exceptions, the compounds were obtained commercially and complied with pharmacopoeial or analytical requirements or both: thus, no specifications are given for them. The following substances were tested.

Phenols. Phenol; *o*-, *m*-, and *p*-cresol; chlorocresol; *o*- and *p*-chlorophenol; *p*-nitrophenol; guaiacol (*o*-methoxyphenol); *p*-methoxyphenol; methyl, ethyl-, and propyl *p*-hydroxybenzoates; also *p*-ethoxyphenol prepared from *p*-phenetidin; the product, recrystallised from water, having a melting point of 65 to 66° (uncorr.) and a solubility of about 0·9 per cent w/v in water at 20°.

Aromatic alcohols. On different occasions commercial samples did not dissolve in water to give clear solutions even when saturation had not been reached. The impurities, presumably traces of hydrocarbons, could not

* This work forms part of a thesis submitted by one of us (H. H.) for the Degree of Doctor of Philosophy in the University of Basle.

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be removed by distillation. The process of purification was carried out as indicated below for 4-chloro-β-phenylethyl alcohol.

The alcohols used were, benzyl alcohol; β-phenylethyl alcohol; γ-phenylpropyl alcohol; phenoxetol (β-phenoxyethyl alcohol); Gecophen (4-chlorophenyl α-glycerol ether). Also included were 4-chlorobenzyl alcohol and 4-chloro-β-phenylethyl alcohol. The chlorobenzyl alcohol was prepared from 4-chlorobenzyl chloride; crystallisation from water gave the required alcohol in colourless needles of a m.p. of 66 to 68° (uncorr.) and b.p. of 116 to 120°/15 mm.; light absorption in ethanol: λ max 266 mμ (log ϵ 2·42); solubility: 0·35 per cent w/v of saline at 20° and 0·30 per cent at 3°. The chlorophenylethyl alcohol was prepared according to Harper and others[1]; purification of the product from traces of water-insoluble compounds was achieved by preparing the monoester of succinic acid and redistilling the saponified product, giving a colourless, virtually odourless liquid; b.p. 80–83°/0·07 mm.; light absorption in ethanol: λ max = 267 mμ (log ϵ 2·55); solubility: 0·48 per cent w/v in water at 20° and 0·46 per cent w/v in saline at 3°.

Organic mercury compounds. Phenylmercuric borate; its solubility is much better in slightly alkaline phosphate solutions; the solution of 0·022 per cent w/v in M/15 phosphate buffer of pH 4 and 5·5 is nearly saturated. Thiomersal.

Quaternary ammonium compounds. Domiphen bromide. Cetyl pyridinium chloride.

Experimental Methods

Buffer solutions. An isotonic phosphate buffer was used since Myers[2] pointed out that different buffer systems would exert different actions on *Escherichia coli*. It consisted of H_3PO_4, plus sodium phosphate with the appropriate amount of KCl added. Although the buffering capacity of this system is small at pH values of 4 and 8·5, it was sufficient for the purpose of the present tests. The phosphates and the phosphorus acid were dissolved under aseptic conditions in freshly distilled water from an all-glass still (Table I).

TABLE I

Preparation of isotonic phosphate buffer solutions

pH	M/15 Phosphorus acid + 0·645 per cent potassium chloride		M/15 Sodium dihydrogen phosphate $NaH_2PO_4 \cdot 2H_2O$ + 0·51 per cent potassium chloride		M/15 Disodium hydrogen phosphate Na_2HPO_4 + 0·38 per cent potassium chloride
4	8 ml.	+	992 ml.		
5·5			940 ml.	+	60 ml.
7			350 ml.	+	650 ml.
8·5*			15 ml.	+	985 ml.

* This solution has a pH of 8·55, after 2 days it falls to about 8·5.

Test solutions. The test solutions were prepared under aseptic conditions by dissolving the disinfectants in the buffer solutions, if necessary on a water bath. The solutions were filled, in amounts of 9 ml., into

aluminium-capped test-tubes, 15 × 160 mm., and inoculated with 1 ml. of bacterial suspension. Stock solutions in distilled water were prepared only from the quaternaries and from thiomersal. Phenols were dissolved in isotonic disodium hydrogen phosphate solution and the pH was adjusted as required. All pH readings were made with a glass:calomel electrode system.

Test organisms. Seven freshly isolated strains of *Escherichia coli* (type I, 44° C.-positive), three freshly isolated and one laboratory strain of *Pseudomonas pyocyanea*, and five freshly isolated strains of *Staphylococcus aureus*, were tested for their resistance to phenol. The most resistant strains were chosen, but results obtained with these were frequently checked with other strains. A culture of the *Staph. aureus* F.D.A. strain (No. 209) at our disposal was not sufficiently resistant and was used only in certain preliminary experiments; other strains also included one gravis and one atypical strain of *Corynebacterium diphtheriae*, one strain of *Serratia marcescens*, and one of *Streptococcus faecalis*.

Nutrient media. Cultures were maintained on agar slopes and sub-cultured every month; from these, new slopes were inoculated every week for test suspensions. The nutrient agar contained 0·5 per cent of peptone (Difco), 0·3 per cent of beef extract (Difco), 0·3 per cent of sodium chloride, 0·2 per cent of desiccated di-potassium hydrogen phosphate, and 2 per cent of agar; the pH was 7·4 to 7·5 before autoclaving at 120° for 20 minutes. The slopes for *C. diphtheriae* were enriched with 0·5 per cent of yeast extract (Difco) and an additional 0·5 per cent of peptone. With *Staph. aureus*, modifications of the nutrient agar did not induce a higher resistance to phenol. The agar was reduced to 1 per cent for the pour-plates and the media used in the disinfection experiments. In these, the nutrient agar was enriched with 0·5 to 1 per cent of yeast extract, an additional 0·5 per cent of peptone, 1 per cent of dextrose, and—when mercurials were tested—0·05 per cent of sodium thioglycollate.

Preparation of the test suspensions. The cultures were grown on slopes for 24 hours at 37° after which they were washed off with 5 ml. of water and the suspensions centrifuged at 3000 r.p.m. for 15 minutes. The centrifuged cells were resuspended in 5 ml. of phosphate buffer, pH 7, and shaken with small glass beads for 5 minutes to break up clumps. This washed suspension was diluted further with buffer so that the cell density in the final test-mixture was about 3×10^5. Plate counts made from each suspension revealed that the variation in the number of viable cells was not more than ± 50,000, and did not affect the results of the disinfection experiments.

The membrane filter technique employed. Cultivation of bacteria on membrane filters was first used for the bacteriological examination of water[3–5]; and subsequently for the testing of skin disinfectants[6–8], as well as for the evaluation of disinfection rates[9]. For the present study, we employed Co 5 membrane filters (Membranfiltergesellschaft, Göttingen) and the corresponding all-metal apparatus. Co 5 filters are nitrocellulose films of 120 μ thickness, the average "pore-diameter"[10] being 500 mμ and the filtering surface 12·5 sq. cm.

For the tests, the filters were "sterilised" in sterile hot water while the tubes with the disinfectant dilutions were placed in a water bath at either 20° ± 0·1° or 37° ± 0·1° half an hour before inoculation with the cell suspensions. After inoculation, the mouth of each tube was flamed and the tubes carefully shaken; this process was repeated once during the longer tests. Cotton-wool plugs are not suitable for the membrane filter method because exact counts cannot be made with accuracy owing to the presence of fibres on the filters. Before filtration, the filter unit was flamed, mounted while hot, and a quantity of sterile water was filtered to cool it. At intervals between 15 minutes and 24 hours (30 minutes, 2 hours, and 24 hours were most currently used), the whole test-mixture was filtered at 1 to 2 ml. per second, after which the filter was washed with 30 to 50 ml. of sterile water. The membrane was then removed and laid on the surface of a prepared nutrient agar plate. The plates were placed in the incubator within 5 minutes and incubated at 37° for 16 to 30 hours before reading. The concentration of agar in the medium was limited to 1 per cent to keep the surface as wet as possible The colonies so developing remain discrete and may be stained by incubating them on nutrient agar containing 0·05 per cent of potassium tellurite[7] for 2 to 4 hours; depending on the species, the colonies are stained brown or black. Counts were made on the dried filters by means of a magnifying lens.

Suitability of the Technique Employed

Influence of the Subculture Medium on the Number of Survivors

Several authors[11–13] have stressed the importance of enriched culture media for cells having survived lethal agents; this opinion, also advocated by Berry and Michaels[14], has not gone unchallenged[15]. We have tested several media with reference to their influence on the survivor count of *E. coli* and *Staph. aureus* by means of the membrane filter method, using phenols, quaternary ammonium germicides and organic mercury compounds and found no significant differences. These media contained various amounts of peptone, yeast extract, meat extract and glucose. The only difference observed was in the rate of growth. We may therefore infer that enriched media are growth-promoting but cannot "revive weakened cells". For this reason a medium containing nutrient agar, 1 per cent of peptone, 1 per cent of dextrose and 0·5 per cent of yeast extract was used in the tests.

Comparison of the Membrane Filter Technique and the Pour-plate Method

A statistical comparison was made with suspensions of *E. coli* and *Staph. aureus* in phosphate buffer pH 7·0 and suspensions of *Ps. pyocyanea* at pH 4·0, 5·5, 7·0 and 8·5. The cell density of the *E. coli* and the *Staph. aureus* suspensions was about 200/ml. and 140/ml. respectively, and the counts were made immediately. For *Ps. pyocyanea* the cell density was about 3×10^5/ml. and the counts were made after storage at 20° for 24 hours. During this time some growth of the organism had taken place, but this is taken into account in the figures recorded in Table II, which shows the comparative counts obtained by the two methods on suspensions

after suitable dilution. These growths probably account for the differences in counts recorded between the duplicate samples, and of the *Ps. pyocyanea* suspension.

TABLE II

COUNTS OBTAINED FROM PARALLEL EXPERIMENTS WITH MEMBRANE FILTERS AND POUR-PLATES

Experiment	Membrane filters (Co 5)				Pour-plates			
	Number of counts	Mean count (x)	Variance (s^2)	Standard deviation (s)	Number of counts	Mean count (x)	Variance (s^2)	Standard deviation (s)
534 (*Staph. aureus*) ..	18	142	90·9	9·5	19	155	221	15
535 (*E. coli*)	20	201	270·1	16	20	206	217·5	15
533 (*Ps. pyoc.*) immediate counts 8·5 a*	10	313	563·9	24	10	307	567·8	24
7 a	5	305	59·75	8	5	319	444·5	21
533 counts after 24 hours at 20° 8·5 a	5	206	461·75	21	6	170	583·6	24
8·5 b	5	70	88·5	9	7	61	13·1	4
7 a	5	129	163·7	13	5	111	193·5	14
7 b	5	95	119·5	11	5	79	51	7
5·5 a	5	102	212·25	15	5	79	150	12
5·5 b	5	92	49·25	7	5	72	92	10
4 a	6	100	149	12	5	113	128·5	11
4 b	4	79	74·3	9	5	88	143·5	12

* a and b are parallel tubes.

TABLE III

SIGNIFICANCE OF THE SUM OF THE PROBABILITIES RESULTING FROM THE t-TEST

Values of t obtained from experiments	Degrees of freedom	P
3·146	35	0·006
1·013	38	0·3
0·564	18	0·6
1·394	8	0·2
2·584	9	0·03
3·989	10	0·005
2·129	8	0·07
2·740	8	0·03
2·702	8	0·03
3·763	8	0·008
1·815	9	0·1
1·257	7	0·3

$\chi^2 = 2\Sigma\ (-\log_e P) = 70{\cdot}50$.
For 24 degrees of freedom (number of P multiplied by 2) $P < 0{\cdot}1$ per cent.

To obtain the counts, 1 ml. amounts were either distributed into petri dishes and plated in the usual way with nutrient agar or they were filtered on a membrane and the membrane was incubated on nutrient agar of the same composition. Each plate or filter was counted three times and individual counts were within $\pm$ 2 to 3 per cent of the mean. From these counts the variances of the two methods were calculated from the F values and they were found to be not significantly different. The t values were also calculated, but from these calculations it was not possible to decide whether the differences in the counts obtained by the two methods were due to chance. Subsequently, the data were subjected to further analyses by means of the combination of probabilities (Fisher[16]), and the findings from these are recorded in Table III. Using the Table of χ^2, we find a probability of less than 0·1 per cent for 24 degrees of

freedom. The difference between the means must therefore be regarded as highly significant. In the experiments with *Ps. pyocyanea*, all the significant differences appeared when the colony counts obtained with the filter method were higher, and it will likewise be seen that the cell density of the suspensions in question is low. From this statistical analysis it may be inferred that membrane filters are slightly superior when suspensions of low cell density have to be counted. This has been shown for *Ps. pyocyanea* with a probability of error of less than 0·1 per cent, but in our experience, it is also true for other test organisms, in spite of Wolochow's[17] opinions to the contrary. With higher counts, Co 5 filters should be rejected in favour of larger filters.

The Variation in Replicate Tests During the Course of Disinfection

Most authors making a quantitative recording of the number of survivors in disinfection experiments have found an excessive variation in replicate plates or roll-tubes[14]. In our experiments, a mortality level of 99·99 to 99·9999 per cent was currently achieved and so a larger set of replicates were filtered to examine in more detail the variations reported. These experiments were made under conditions as identical as possible, for example, using one test suspension for each series, the same batch of nutrient agar, membranes of the same serial number, and the disinfectant solutions were readjusted to pH 7·0. The initial counts were as usual $3 \cdot 10^5$ cells/ml., and 10 ml. amounts were filtered. Parallel with the larger series, two smaller ones were made also, representing the corresponding routine tests made in triplicate: the findings are summarised in Figures 1–4. Each of the Figures shows a strong divergence from a Poisson series, and this is the type of variation which has been encountered throughout this present study. It has compelled us to represent the results by means of a graphical method in which "mean counts"—represented by dotted lines—were computed from the logarithms of the single counts.

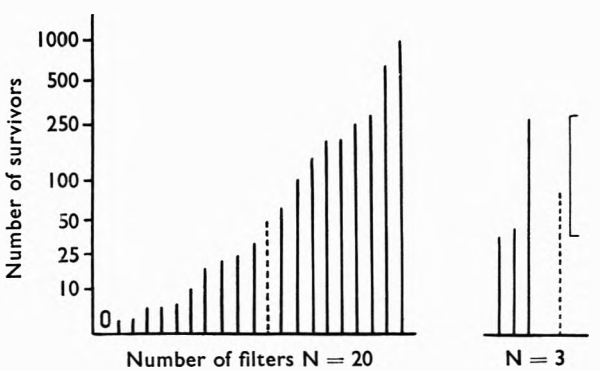

FIG. 1. Membrane filter counts of suspensions of *E. coli* in parallel tubes exposed for 2 hours at 20° to 0·3 per cent of *p*-cresol in isotonic phosphate buffer with a pH of 7·0. Left: Results of a larger series. Right: Results of a routine test. Dotted lines = "means", calculated from the logarithmic values. Each column represents the survivors of 10 ml. of test-mixture with an initial count of $3 \cdot 15^5$ cells/ml., 0 = sterile.

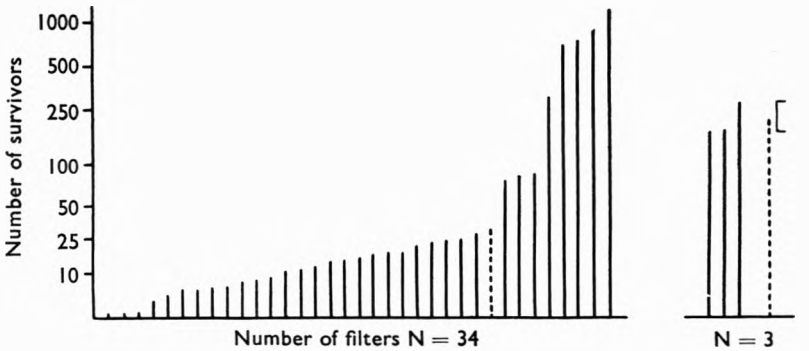

FIG. 2. Membrane filter counts of suspensions of *Staph. aureus* exposed for 24 hours at 20° to 0·2 per cent of 4-chloro-β-phenylethyl alcohol in isotonic phosphate buffer with a pH of 7·0. Left: a larger set of parallel filtrations was made. Right: Records the results of a corresponding routine test. Cell density and representation as in Fig. 1.

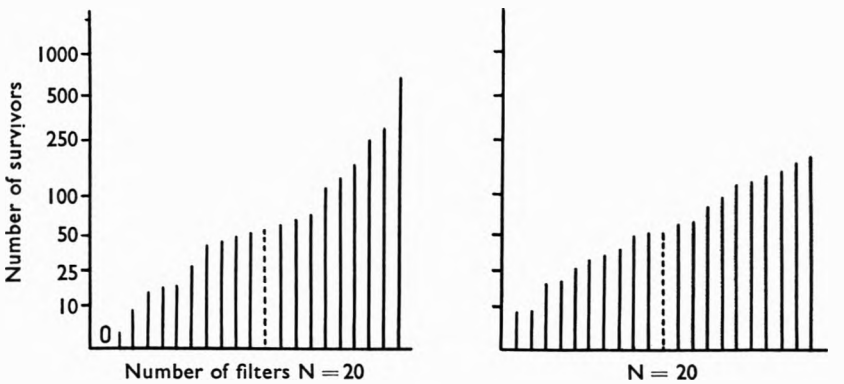

FIG. 3. Membrane filter counts of suspensions of *E. coli* (Left) and *Staph. aureus* (Right) exposed, for 30 minutes at 20°, to solutions of domiphen bromide 1 : 50,000 in isotonic phosphate buffer with a pH of 7·0. Cell density and representation as in Fig. 1.

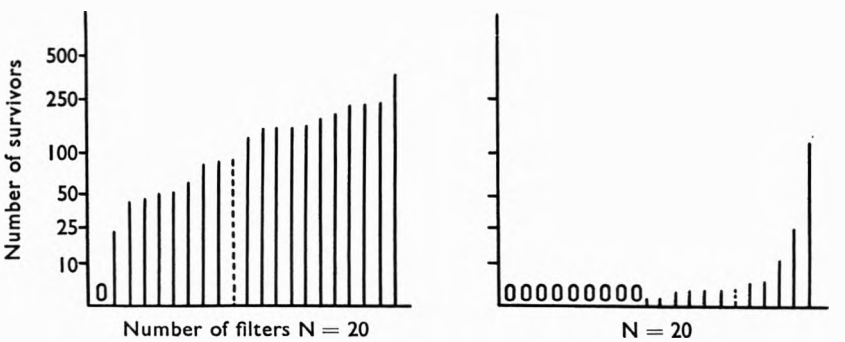

FIG. 4. Membrane filter counts of suspensions of *E. coli* (Left) and *Staph. aureus* (Right) exposed, for 30 minutes at 20°, to cetyl pyridinium chloride 1 : 100,000 in isotonic phosphate buffer with a pH of 7·0. Cell density and representation as in Fig. 1.

Viability of the Test Organisms in the Buffer Solutions

Suspension of the different organisms used were tested for their viability at different pH values at 20° for up to 24 hours; the number of viable cells was determined by plating, and sometimes parallel counts were made by means of membrane filters. Only the essential data are given here; for detailed results, see reference 18.

At a pH of 4, the mortality of *Ps. pyocyanea* was about 70 per cent and that of *E. coli* even less. Moderate growth occurred at the other pH values, with exception of *E. coli* at pH 8·5 where the viable count remained constant. *Staph. aureus* and *C. diphtheriae* were very sensitive to an acid pH; at pH 4, the suspensions were almost non-viable after 24 hours. The freshly isolated strains of *Staph. aureus*, used in the tests because of their higher resistance to phenol, were more easily killed by an acid pH than the F.D.A. strain at our disposal. At pH 7 and 8·5, the number of viable Gram-positive cells remained approximately constant.

Discussion

Statistical analysis has shown that the membrane filter technique adopted here is reliable when used to evaluate comparatively small numbers of viable cells in suspensions. The use of filters in disinfection experiments therefore has several obvious advantages: any quantity of test-mixture may be filtered, the filters may be washed to eliminate the danger of bacteriostat carry-over, except for mercurials, and colony growth occurs under uniform aerobic conditions.

The sensitivity of the membrane filter method is reflected in the results obtained with the three cresols. These are said to have equal bactericidal activity when measured with the phenol coefficient[19]. We have found, however, that *p*-cresol was most effective, closely followed by *o*-cresol, *m*-cresol being much less effective. The difference obtained can be ascribed only partly to the longer period of exposure, namely 30 minutes.

The disadvantages of end point methods in assessing disinfectants have been summarised by Berry[20], Berry and Michaels[21], Withell[22,23], and Wilson and Miles[24]. Nevertheless, in recent papers[25,26], end point methods have still been advocated because quantitative tests are greatly hindered by agglutination of the test suspension, and sterility has always been the desirable state. As for the counting methods, the mortality levels used therein have varied greatly. Jordan and Jacobs[27] preferred a very high mortality level of 99·999999 per cent ("virtual sterilisation time"), starting from an initial count of $3{\cdot}3 \times 10^8$. Other workers have suggested 99·9 or 99 per cent killing levels. Withell[22] introduced an LT50, the advantage being that bacteriostasis is avoided to a large extent in the subculture, and also that excessive variation in replicate tests is said to be reduced thereby. Jordan and Jacobs[27,28] found most discrepancies in their results when the mortality exceeded 95 per cent. Berry and Michaels[14] also observed some excessive variation in their disinfection experiments, even when using intermediate mortality levels, whereas for cells not having been in contact with disinfectants the distribution of

replicate counts followed a Poisson series. They tried to explain the difference by assuming that damaged organisms had more exacting growth requirements, and then went on to quote several authors in support of their thesis. As another possible explanation, Jordan and Jacobs have suggested that a certain percentage of the cells already damaged might be killed by the temperature of melted agar during the preparation of the plates or roll-tubes. Every heterogeneity of this type in colony formation would increase the distribution error already present and thus cause a rise in variation. These authors therefore suggested that a more adequate technique be adopted to handle the larger variation.

The membrane filter technique appears to be the answer to this problem. It yielded satisfactory results when untreated cells were counted; the danger of heat bacteriostasis is excluded since no heat is used, and, finally, growth takes place under equal conditions for all cells. Moreover, by filtering the whole test-mixture, the distribution error can be greatly reduced.

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References

1. Harper, Reed and Thompson, *J. Sci. Food Agr.*, 1951, **2**, 94.
2. Myers, *J. Bact.*, 1928, **15**, 341.
3. Schütz and Kruse, *Zbl. Bakt. I Orig.*, 1947–8, **152**, 135.
4. Beling and Maier, *Vom Wasser*, 1954, **21**, 118.
5. Clark and Kabler, *Amer. J. Publ. Health*, 1952, **42**, 385.
6. Hitsch, *Schweiz. med. Wschr.*, 1950, 1285.
7. Naumann, *Z. Hyg.*, 1952, **135**, 161.
8. Brömmelhues, *Medizin und Chemie, Abh. Bayer*, 1956, **5**.
9. Uehleke and Poetschke, *Zbl. Bakt. I Orig.*, 1957, **168**, 141.
10. Henneberg and Crodel, *ibid.*, 1954, **160**, 605.
11. Curran and Evans, *J. Bact.*, 1937, **34**, 179.
12. Nelson, *ibid.*, 1943, **45**, 395.
13. Nelson, *ibid.*, 1944, **48**, 473.
14. Berry and Michaels, *Quart. J. Pharm. Pharmacol.*, 1947, **20**, 331.
15. Hershey, *J. Bact.*, 1939, **38**, 563.
16. Fisher, *Statistical Methods for Research Workers*, 12th Edn., Oliver and Boyd, Edinburgh, 1954.
17. Wolochow, *Appl. Microbiol.*, 1958, **6**, 201.
18. Hess, Thesis University of Basle, 1958.
19. Klarmann and Shternov, *Industr. Engng Chem.* (Anal.), 1936, **8**, 369.
20. Berry, *J. Pharm. Pharmacol.*, 1951, **3**, 689.
21. Berry and Michaels, *ibid.*, 1950, **2**, 243.
22. Withell, *J. Hyg., Camb.*, 1942, **42**, 339.
23. Withell, *Quart. J. Pharm. Pharmacol.*, 1942, **15**, 301.
24. Wilson and Miles, Topley and Wilson's *Principles of Bacteriology and Immunity*, 4th Edn., Edward Arnold, London, 1955.
25. Berry and Bean, *J. Pharm. Pharmacol.*, 1954, **6**, 649.
26. Cook and Wills, *ibid.*, 1954, **6**, 638.
27. Jordan and Jacobs, *J. Hyg., Camb.*, 1944, **43**, 275, 363.
28. Jordan and Jacobs, *ibid.*, 1945–6, **44**, 210.

A SLOPING SCREEN METHOD FOR THE BIOASSAY OF INSULIN IN MICE

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A quantal response method for the bioassay of insulin is described employing a sloping screen for the detection of hypoglycaemic symptoms in insulin-treated mice. This procedure tends to eliminate the personal bias which may occur in assays using convulsive seizures or a state of collapse as the criterion of the response to insulin. In a comparison of this method with that described for insulin in the British Pharmacopoeia[1], it was found that although the slopes of the log dose-response lines did not differ significantly, the procedure using the sloping screen for detecting the response required a larger dose of insulin. Mice primed with 5 mU. of insulin before their routine use in assays were more uniform in their response than unprimed mice. The presence of a retarding agent such as gelatin or protamine added to insulin did not influence the slope of the log dose-response line, but may under special conditions delay the absorption of insulin from the injection site. Evidence has been obtained which suggests that the amount of daylight to which the mice are exposed may have a significant effect on the precision of the assay.

The qualitative effect of the administration of insulin to laboratory animals may be observed either by measuring the fall in blood sugar or by the incidence of hypoglycaemic symptoms such as convulsions or muscle weakness. This response to insulin can be relieved by the administration of glucose.

Several procedures have been described for detecting hypoglycaemic reactions to insulin in mice[2]. An elevated temperature was employed in earlier studies to induce the characteristic convulsive seizures in insulin-treated mice. However in these assays trained personnel were required to recognise the symptoms attributable to insulin and to administer glucose quickly to prevent fatalities among the stricken mice. Thompson[3] found that mice showing a reaction to insulin would fall off a wire-mesh screen set at an angle of 60°. This type of equipment for the objective determination of the presence or absence of advanced insulin symptoms tended to eliminate personal bias and reduced the number of personnel required to perform an insulin assay. Young and Lewis[4] modified this apparatus by using revolving wire-mesh drums instead of the flat screens. The affected mice lost their foothold while the drum was turning and fell into trays containing food, the consumption of which was sufficient to alleviate the hypoglycaemia.

In the method of assay described in this communication, a modification of Thompson's[3] procedure for detecting the response to insulin has been employed, and various factors affecting this type of assay have been investigated.

Experimental

Female albino mice, raised in our own colony and weighing 18 to 24 g., are kept in cages in a room maintained at 27° ± 1° and are given Master Fox cubes* and water *ad libitum*. On the day of an assay, the food is withdrawn at 8.30 a.m., and the mice are weighed and distributed in containers according to their weight. All the mice in a single container have the same weight. Either 120 or 160 mice within a weight range of 4 to 5 g. are selected from the containers in such a way that the number from each weight group included in each of four dosage groups is the same. Thus the average weight of the mice is similar in each dose group.

The insulin diluent, unless otherwise specified, is an aqueous solution containing 0·90 per cent sodium chloride, 0·15 per cent phenol, and sufficient hydrochloric acid to adjust the acidity of the solution to pH 2·5 to 3·0. The stock insulin solution is prepared from the International Standard or the insulin preparation under test to contain 1·0 I.U./ml., and suitable dilutions are made from this stock solution. A volume of 0·20 ml., containing the desired dose of insulin, is injected subcutaneously into the mid dorsal area of each mouse. The log dose interval is either 0·2218 or 0·3010.

The mice are starved for a period of 4 to 5 hours, injected with the diluted insulin, and then placed on a sloping screen set at an angle of 60°. The apparatus used in this work is a modification of that originally described by Thompson[3]. It consists of four compartments, 12 in. × 25 in. in size, of aluminium window-screening, and is set up in the laboratory in which the mice are housed. The framework holding the screens is also made of aluminium and is placed on a laboratory bench with the lower edge of the screens extending over the edge of the bench-top. The distance to the floor is 30 in. On rare occasions mice fall or jump from the screen at the beginning of the assay. To avoid false-positive responses, any mouse that falls off the screen during the first 20 minutes after the injection is replaced on the screen and watched carefully. Experience has shown that if the mouse falls again within a few minutes it is displaying a true hypoglycaemic response. After the initial 20 minutes' period of the assay, the mice appear to lose interest in exploring the lower part of the screen and usually remain clinging to one place for the remainder of the assay. The mice showing a positive response to the insulin fall into metal containers, partially filled with sawdust, which are placed immediately below each of the 4 screens. The affected mouse is usually given 0·5 to 1·0 ml. of a 5 per cent solution of glucose, and is permitted to recover from the hypoglycaemic reaction in a cage containing food and water. By using this technique very few of the mice die during an assay. The mice are left on the screen for a period of 90 minutes after the injection, and the number of mice remaining in each dose group is recorded at that time. The assay is carried out in the "mouse room" to avoid any change in the environmental temperature.

* Master Fox cubes are available from Toronto Elevators Ltd., Toronto, Canada.

Since there are 4 dosage groups, a 2 × 2 design is used, and the potency ratio and its confidence limits are calculated by the quantal response method using probits[5–7]. Each assay is checked for validity by suitable χ^2 tests. To increase the precision, 4 to 8 assays are usually combined by "method C" described by Perry[8]. This procedure permits, in addition to the calculation of the mean potency ratio and its confidence limits, the estimation of the mean slope as well as the average median effective dose and its standard error[7].

Results and Discussion

Comparison of the Sloping Screen Method with the Procedure Described in the British Pharmacopoeia[1]

A glass-fronted air incubator was used to determine the potency of the Third International Standard for Insulin by the method described in the British Pharmacopoeia[1] for the biological assay of insulin injection. The cabinet was maintained at 30 ± 1° during the assay period of 90 minutes. The mice were starved overnight, given a subcutaneous injection of the dilute insulin solution and placed in litre beakers in the cabinet. A total of 96 mice were used in a 2 × 2 assay design. The log dose interval was 0·3010. When a mouse displayed convulsive seizures or passed into a state of collapse, it was removed from the beaker in the cabinet and given an injection of a 5 per cent solution of glucose. The number of mice affected in this way by the insulin was recorded, and the potency ratio calculated by the probit method[7].

The sloping screen procedure was employed to assay the Fourth International Standard for Insulin, and the results are compared in Table I

TABLE I

Comparison of the sloping screen method with that described in the British Pharmacopoeia[1]

Method	Average slope of the log dose-response line b	Average median effective dose ED50 ± S.E.**	Potency of International standard for insulin	
		mU.	Found I.U./mg.	Adopted I.U./mg.
British Pharmacopoeia[1] air incubator at 30°C. (6 assays)	4·13 ± 0·57*	13·4 ± 0·5	25·6 (22·1-29·6)	24·5
Sloping screen at 27°C. (9 assays)	3·77 ± 0·30	15·6 ± 0·5	24·2 (21·7-26·9)	24·0

$$* \sqrt{Vb} = \frac{1}{\sqrt{[nwx^2]}}$$

** S.E. = $S_{\log ED50} \times 2{\cdot}303 \times ED50$ (7)

with those obtained with the method described in the British Pharmacopoeia[1]. Both assay procedures provided an estimate of the potency which did not differ significantly from that adopted after the completion of the collaborative assay to establish the biological activity of the International Standard Insulin preparations.

Although the values for the slopes (b) of the log dose-response lines estimated for the 2 assay methods are not the same, their standard errors suggest that this difference could occur by chance alone. However the median effective dose (ED50) calculated for the sloping screen procedure tends to be larger than that estimated for the method described in the British Pharmacopoeia[1]. Possibly the lower ED50 and therefore the increased sensitivity can be attributed at least in part to the higher temperature to which the mice are subjected in the latter procedure.

Effect of a Priming Dose of 5 mU. of Insulin on the Precision of the Assay

Mice employed in an assay for the first time frequently are more sensitive and behave more erratically than those which have been used in at least one test previously. Since it is necessary to include new mice from the stock colony from time to time to make up the quota of 120 to 160 mice for an assay, this apparent difference in sensitivity can lead to invalid assays or heterogeneity between the estimates of the potency ratios when the assays are combined.

Accordingly a study was made of the effect of giving the new mice a priming dose of 5 mU. of insulin before they were included in a regular assay. The individual estimates of the median effective dose for the unprimed mice were found to be heterogeneous when they were tested by χ^2, and semi-weights estimated by the method of Bliss[9] had to be used to calculate the average median effective dose for these mice. The results in Table II indicate that the ED50 for the unprimed mice was subject not

TABLE II

EFFECT OF A PRIMING DOSE OF 5mU OF INSULIN ON THE ED50 AND THE SLOPE OF THE LOG DOSE-RESPONSE LINE

Treatment of mice	No. of assays	Average ED50 ± S.E. mU.	Average slope
Unprimed	4	11·7 ± 5·5	3·20 ± 0·55
Primed with 5 mU of insulin	5	16·5 ± 1·1	3·33 ± 0·54

only to more variability as shown by the 5-fold larger standard error, but was also smaller than that for the primed mice. The values for b given in Table II are not significantly different, suggesting that priming of the mice has no real effect on the slope of the log dose-response line. As a result of this study, mice obtained from the stock colony are routinely given a dose of 5 mU. of insulin and placed on the sloping screen for a period of 90 minutes a few days before they are included in a regular insulin assay.

Effect of the Injection Medium on the Response of the Mice to Insulin

Various retarding agents were added to the acid saline injection medium in an attempt to improve the precision of the assay by increasing the slope of the log dose-response line. The materials employed are listed in

Table III together with the values for the slope of the log dose-response line and the median effective dose. The influence of the retarding agent on the relative potency is also shown in Table III. The acid saline injection medium was used as the reference standard in each of these assays.

TABLE III

EFFECT OF THE INJECTION MEDIUM ON THE RESPONSE TO INSULIN

Type of insulin preparation	No. of assays	Retardant, per cent	Slope of the log dose-response line		χ^2_b	Relative potency medium alone = 100 per cent P = 0·95
			Medium alone	Medium + retardant		
Cryst. insulin	4	carboxy-methylcellu-lose 1	4·22 ± 0·67	3·31 ± 0·56	1·08	142·9 (106·5–191·5)
Cryst. insulin	4	polyvinyl-pyr-rolidone 2	4·00 ± 0·62	4·91 ± 0·61	1·10	98·2 (86·0–112·0)
Cryst. insulin	1	pectin 3	5·23 ± 1·25	2·30 ± 1·10	3·10	77·6 (55·0–109·6)
Cryst. insulin	6	gelatin 16	3·87 ± 0·50	2·69 ± 0·46	3·01	96·1 (82·9–111·5)
Isophane insulin ..	5	gelatin* 16	2·45 ± 0·51	3·57 ± 0·60	2·03	56·2 (45·2–69·7)
Protamine zinc insulin	2	protamine* (1 mg./ml.)	4·02 ± 0·85	3·06 ± 0·81	0·67	83·8 (64·4–109·1)

* Injection medium at pH 7·2.

Although the values for b vary, the test for χ^2_b was not significant in any of the assays indicating that the presence of the retarding agent in the medium did not affect the slope to any extent. It is difficult to understand why the addition of 1 per cent carboxymethylcellulose to the medium enhanced the insulin effect. Perhaps the carboxymethylcellulose protected the insulin from tissue proteases at the injection site. Neither polyvinylpyrrolidone, pectin, nor gelatin, at the concentrations employed, had a significant effect on the relative potency. No delay in the onset of hypoglycemic symptoms was observed in the test animals when any one of these agents was added to the acid saline injection medium. Apparently at the dilution required in the mouse assay, insulin does not readily form a complex with any of the retarding agents employed in this study. The insulin probably occurred in the free state in the injection medium. In contrast, when isophane insulin was diluted with 16 per cent gelatin at pH 7·2, the crystalline protamine-zinc-insulin complex must have been partially dissociated only because little more than 50 per cent of the activity was available to the mice. This is probably not an effect of the hydrogen ions because the addition of excess protamine to protamine zinc insulin at pH 7·2 did not reduce the relative potency of the preparation.

This work supports the observation made earlier by Young, Reid, and Romans[10] that the mouse convulsion method of assay is a satisfactory procedure for determining the insulin content of commercial preparations such as protamine zinc insulin and globin insulin with zinc. Excellent results have been obtained in this laboratory by using the sloping screen method for determining the insulin content of protamine zinc insulin, isophane insulin, globin insulin with zinc, and insulin zinc suspension.

Effect of Daylight on the Response of the Mice to Insulin

A survey of the average slopes of the log dose-response lines obtained in routine insulin assays over a period of several years revealed that the values tended to be higher during the winter months than they were during the summer. Since the mice were kept in air-conditioned quarters, this variation could not be attributed to changes in environmental temperature.

TABLE IV

INFLUENCE OF LIGHT ON THE RESPONSE OF MICE TO INSULIN

Time of year	No. of assays	Slope of log dose-response line b_s	Median effective dose of standard ED50mU ± S.E.	Remarks
June 1956 ..	5	2·83 ± 0·46	42·6 ± 2·8	Bright light, no blinds on windows
August 1956 ..	12	4·57 ± 0·36	18·7 ± 0·5	Dark, covers over windows, room lights on during assays
December 1957	4	5·10 ± 0·78	16·4 ± 0·6	Difluse light, venetian blinds over windows
June 1958 ..	6	3·57 ± 0·63	21·8 ± 1·1	Diffuse light, venetian blinds over windows
December 1958	7	6·16 ± 0·69	16·2 ± 0·4	Diffuse light, venetian blinds over windows
May 1959 ..	4	4·13 ± 0·92	22·1 ± 1·5	Covers over windows, lights on from 8 a.m. until 5 p.m.
June 1959 ..	4	4·20 ± 0·80	20·2 ± 1·0	Covers over windows, lights on from 8 a.m. until 5 p.m.

According to Table IV, in June, 1956, the slope of the line for the International Insulin Standard was only 2·83 ± 0·46, while in December, 1957 and 1958, it was 5·10 ± 0·78 and 6·16 ± 0·69 respectively. Also the highest value for the median effective dose was obtained in June, 1956, while the lowest values were observed in December, 1957 and 1958. During June, 1956, the mouse room was exposed to daylight all day, but in July, 1956, the windows were covered with cardboard and the lights were turned on in the mouse room during the period of the assay only. The data in Table IV indicate that the slope of the line estimated during August, 1956, was significantly steeper than that calculated in June, 1956, and the median effective dose was closer to that found during the winter months. Eventually venetian blinds replaced the cardboard covers and this change permitted a diffused light to enter the mouse room during the daylight hours. These conditions prevailed in June, 1958, and it will be seen that the average slope tended to be lower than it was in August, 1956. In May, 1959, the cardboard covers were replaced on the windows and the lights were turned on at 8.0 a.m. and off at 5.0 p.m. by a time switch. The slopes found under these conditions were slightly higher than that obtained in June, 1958, but the difference was not actually significant.

Although these data are by no means complete, there is enough evidence to suggest that prolonged exposure to daylight reduces the precision of the assay. Mice appear to be more resistant to the insulin and do not differentiate between the dose levels as well as they do when kept in the dark for longer periods. Apparently the seasonal variation in the steepness of the log dose-response line which was observed with our mice can

be explained on the basis of the amount of daylight which entered the mouse room at the various times of the year. Perhaps the activity of the mice, which is greater in subdued light or darkness than it is in daylight, is one of the factors responsible for this effect of light on the precision of the assay.

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References

1. *British Pharmacopoeia*, 1958, p. 935.
2. Smith, in *Hormone Assay*, edit. Emmens, Academic Press Inc., New York, 1950, p. 35.
3. Thompson, *Endocrinology*, 1946, **39**, 62.
4. Young and Lewis, *Science*, 1947, **105**, 368.
5. Miller, Bliss and Braun, *J. Amer. pharm. Ass.*, 1939, **28**, 644.
6. Miller, *ibid.*, 1944, **33**, 245.
7. Finney, *Probit Analysis*, 1st Edn., Cambridge University Press, 1947.
8. Perry, *The Design of Toxicity Tests*, Reports on Biological Standards, Medical Research Council, Special Report Series No. 270, London, 1950.
9. Bliss, *Drug Standards*, 1956, **24**, 33.
10. Young, Reid and Romans, *Can. J. Research*, 1950, **28E**, 19.

THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURY COMPOUNDS

PART I. THE ANTIBACTERIAL ACTIVITY OF MERCURIC CHLORIDE

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E. coli I was the most resistant of nine organisms tested to the bacteriostatic action of mercuric chloride. Incubation of peptone water or Lemco broth containing high concentrations of mercuric chloride produced a greyish-black sediment. The inclusion of dextrose in the medium increased the amount of mercuric chloride necessary for bacteriostasis. The bacteriostatic values of the liquid and solid dilution methods differed, and the results of the liquid dilution method could be varied by alteration of the experimental technique. The bacteriostatic activity of mercuric chloride was greater at 37° than at 20°. The presence of culture medium has a protecting action on the organisms. Mercuric chloride prolongs the lag phase of *E. coli* I but its mechanism has not been investigated.

As few quantitative data are available on the antagonism of the antibacterial action of mercury compounds, a study of their antagonism by various sulphydryl-containing materials was undertaken. The mode of action of mercury compounds on bacteria and some of the factors which could affect this were first investigated. Although several mercury compounds, both inorganic and organic, are in use, the one selected for the work described in this series of papers was the simplest, mercuric chloride. Preliminary experiments showed that of the nine organisms used by Cook and others[1], *E. coli* I was the most resistant to the bacteriostatic action of mercuric chloride.

EXPERIMENTAL

Mercuric chloride. Analytical reagent was used. Stock solutions were prepared with freshly boiled and cooled distilled water, and stored in the dark. These were assayed by the method of the British Pharmacopoeia 1953. Dilutions for use were prepared when required.

Dropping pipettes. These were of the type described by Cook and Yousef[3]. Four needles were calibrated and the 95 per cent confidence limits of the weight of single drops of water delivered were 17·14 to 17·20 mg., thus the volume of one drop of water from any of the needles may be taken as 1/58 ml. The possible change in drop-weights delivered by the needles after prolonged usage may be neglected since after 24 months use, during which period they had been sterilised numerous times by autoclaving, boiling and dry heat, the confidence limits of a single drop of water delivered from any of the needles were 17·08 to 17·14 mg.

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Media. The liquid medium (peptone water) contained 1 per cent of Oxoid peptone and 0·5 per cent of sodium chloride. It was prepared with water distilled from a stainless steel still, and the reaction of the medium was adjusted to pH 7·2. For some experiments the medium was prepared double strength. The solid medium (peptone agar) was of the same composition, being gelled by the addition of 1·5 per cent of Davis bacteriological agar. The Lemco broth consisted of peptone water fortified by the addition of 1 per cent of Lab-Lemco. All media used during the course of this work was prepared from the same batch of peptone.

Test organism. A laboratory strain of *Escherichia coli* type I, formerly N.C.T.C. No. 5933 was used. It was maintained by the continuous daily subculture method described by Cook and Wills[4].

Methods. The liquid and solid dilution methods for evaluating bacteriostatic activity were used as described by Cook[5].

Both methods were carried out simultaneously in replicate with controls. Typical results for the bacteriostatic value of mercuric chloride gainst *E. coli* I were 70 and 150 μM respectively by the liquid and solid dilution method.

No significant difference in the bacteriostatic value of mercuric chloride was noted between the results obtained by the liquid dilution method when peptone water or Lemco broth was used. At high concentrations of mercuric chloride (about 0·1 per cent) a greyish-black sediment appeared in the tubes of media, both inoculated and uninoculated, after incubation; precipitation occurred to a greater extent with the Lemco broth than with the peptone water.

Comparative determinations of the bacteriostatic value using peptone water with and without 1 per cent of dextrose gave results of 75 and 60 μM of mercuric chloride respectively for the two media.

To keep the systems as simple as possible, without resorting to chemically defined media, peptone water was used as the medium for all the work involving *E. coli* I.

It was noted that by modification of the liquid dilution method considerably different results for the bacteriostatic value of mercuric chloride could be obtained. As usually performed, the liquid dilution method involves mixing double strength culture medium with an equal volume of bacteriostat solution before addition of the inoculum. By addition of the inoculum to the bacteriostat solution and maintaining for a period before adding the culture medium, an apparently much lower concentration of mercuric chloride was required for bacteriostasis. The experiments described below were carried out simultaneously and with replication, with the following results for mean bacteriostatic concentrations. A. The liquid dilution method[5]: 63 μM. B. The inoculum was added to 5 ml. of mercuric chloride solution and allowed to stand at 20° for 1 hour before adding the double strength culture medium and incubating at 37°: 17 μM. C. as in B but keeping the reaction mixture at 37°: 10 μM. D. The inoculum was added to 1 ml. of mercuric chloride solution and allowed to stand at 20° for 1 hour before adding 5 ml. of double strength culture medium and 4 ml. of water, and incubating at 37°: 5 μM. E. As in D but keeping the reaction mixture at 37°: < 1 μM.

In the light of these findings that the bacteriostatic value of mercuric chloride against *E. coli* I varied with the method of test used, the liquid dilution technique for determination of bacteriostatic value was carried out by the published method[5], except where specifically indicated otherwise.

Effect of Mercuric Chloride on the Lag Phase of E. coli *I*

One drop of a 24-hour culture of the organism was added to each of a series of tubes of peptone water containing increasing concentrations of mercuric chloride, and the inoculated mixtures incubated at 37°. The time taken for visible growth to occur was noted and the mean values for six replicates are shown in Table I.

On further incubation the inoculated mixtures all eventually reached approximately the same optical density, except those containing a bacteriostatic concentration of mercuric chloride.

Discussion

The bacteriostatic value of mercuric chloride against *E. coli* I as determined by the solid dilution method was approximately double that by the liquid dilution method. Cook[5] showed a correlation of the two methods for evaluating bacteriostats, but his experiments were conducted with ten-fold dilutions of the bacteriostats and not such close concentrations as used here.

The reason for the discrepancy in the values obtained with *E. coli* I by the two methods remains obscure and it is possible that it might not have been noted if a wider range of dilutions had been used. Several explanations appear feasible: (*a*) organisms on the surface of the medium in the solid method are not in such intimate contact with the bacteriostat as they are in the liquid method and hence a higher concentration of bacteriostat may be required for bacteriostasis; (*b*) the work of Bean and Walters[6] on the release of nitrogenous materials from killed cells of *E. coli* which were capable of supporting the growth of living cells may be pertinent in this phenomenon; cells in immediate contact with the bacteriostat in the solid medium may release these materials which serve to increase the resistance of cells in not such close contact with the bacteriostat; (*c*) combination of mercuric chloride with constituents of the medium may occur to a greater extent in the solid medium, which could be significant by reducing the effective concentration of mercuric chloride or by rendering essential nutrients unavailable to the organisms; (*d*) the absorption of a drop of liquid medium into the surface of solid medium containing bacteriostat may cause a local decrease in the effective concentration of the bacteriostat in the drop area; (*e*) volatilisation of the mercuric chloride[2] may occur on mixing the bacteriostat solution with the molten peptone agar in the solid dilution method.

The precipitation occurring in peptone water containing mercuric chloride was noted by Hotchkiss[7] and is believed to arise from the reaction of the mercuric chloride with constituents of the medium. Greater reaction with Lemco broth would therefore be expected.

Dextrose has been reported to be without effect upon the antibacterial activity of mercuric chloride[8]. The decrease in the bacteriostatic activity of mercuric chloride observed in the presence of dextrose can be simply explained on the grounds that inclusion of a fermentable carbohydrate provides a more nutrient medium for the growth of the organisms and one in which they are less susceptible to adverse conditions. The efficiency of mercuric chloride is increased by a reduction in pH[9]; fermentation of dextrose will reduce the pH of the medium to about pH 4 to 5 which should increase the activity of the mercuric salt. That more mercuric chloride is required in the presence of dextrose to produce bacteriostasis implies either that mercuric chloride is less active against actively multiplying cells or that the inoculum has increased to a value greater than the critical value for a given concentration of mercuric chloride[10].

Some lowering of the oxidation-reduction potential of the system may occur in the presence of dextrose but this factor was not investigated. No antagonism of the mercuric chloride by the dextrose is envisaged.

TABLE I

TIME TAKEN FOR VISIBLE GROWTH OF *E. coli* I IN PEPTONE WATER CONTAINING MERCURIC CHLORIDE

Concentration of mercuric chloride	Time for visible growth
Nil	2·5 hours
5 μM	2·5 "
10 "	2·6 "
15 "	3·0 "
20 "	3·0 "
25 "	4·4 "
30 "	4·8 "
35 "	5·5 "
40 "	8·0 "
45, 50, 55 "	>8 "
60 " (b'static concn.)	∞

The varied bacteriostatic values for mercuric chloride when determined by different methods have been interpreted as follows.

(i) In methods B, C, D and E the organisms do not have the protecting influence of the medium constituents until after the mercuric chloride has been adsorbed to them.

(ii) In the modified methods, there is no chance of reaction of the mercuric chloride with the medium constituents before the bacteria are "coated" with the mercurial salt, and thus the effective concentration of the mercuric chloride is not decreased until after it has begun to exert its antibacterial action.

(iii) In the absence of the medium in the modified methods, the mercuric chloride can enter into a stable combination with the organisms which is not reversed upon subsequent addition of the medium.

(iv) The increased efficiency of mercuric chloride in methods C and E as compared with that in B and D is merely a function of the temperature at which the reaction mixtures were held; indicating that mercuric chloride is a more efficient bacteriostat at 37° than at 20°.

(v) The increased efficiency of mercuric chloride in methods D and E over that in B and C may be explaind by the fact that the binding of sulphydryl groups by mercury is a second order reaction[11]; from this it is inferred that the speed of reaction decreases with increasing dilution and hence a higher concentration of mercuric chloride is required to produce bacteriostasis in a given time.

The long lag period after the use of mercurial compounds has been noted by many workers. In demonstrating the effect of mercuric chloride

on the lag phase of the organism (Table I) the whole interval between inoculation and the first visible signs of growth is not the lag period, but these results may be regarded as an indication that mercuric chloride prolongs the lag phase or retards the rate of multiplication of *E. coli* I, or both. To show which of these two factors is mainly responsible the experiment could be carried out quantitatively, following the growth by a series of total and viable counts.

In later work, the isolation of a strain of *E. coli* I more resistant to the antibacterial action of mercuric chloride was attempted. The procedure consisted of subculturing the organism daily, or on alterante days, into peptone water containing increasing concentrations of mercuric chloride. The time taken for visible growth to occur increased with increasing concentration of mercuric chloride, but once the organisms had become adapted to growth in a particular concentration of the mercuric salt, further subculture into the same concentration resulted in growth appearing more rapidly.

References

1. Cook, Steel and Wills, *J. Pharm. Pharmacol.*, 1956, **8**, 721.
2. Bellucci and Casini, *Ann. Chim. appl., Roma*, 1951, **41**, 374.
3. Cook and Yousef, *J. Pharm. Pharmacol.*, 1953, **5**, 141.
4. Cook and Wills, *ibid.*, 1956, **8**, 266.
5. Cook, *ibid.*, 1954, **6**, 629.
6. Bean and Walters, *ibid.*, 1955, **7**, 661.
7. Hotchkiss, *J. Bact.*, 1923, **8**, 141.
8. Salle and Ginoza, *Proc. Soc. exp. Biol., N.Y.*, 1943, **54**, 85.
9. Gershenfeld and Perlstein, *Amer. J. Pharm.*, 1941, **113**, 237.
10. Poole and Hinshelwood, *J. chem. Soc.*, 1940, 1565.
11. Rapkine, *J. Chim. phys.*, 1936, **33**, 493.

FURTHER OBSERVATIONS ON THE BIOLOGICAL PROPERTIES OF DEQUALINIUM (DEQUADIN) AND HEDAQUINIUM (TEOQUIL)

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The activity of dequalinium and hedaquinium against *Staphylococcus aureus* and *Candida albicans* is strongly antagonised by the sodium salts of fatty acids, but not by a number of detergents and a commercial liquid soap. Sufficient antagonism was shown by agar to render invalid the results obtained in assays of these antimicrobial agents by the agar plate method. Human saliva had only a slight antagonistic action on both substances. Twenty-one strains of *Candida albicans* showed some variation in sensitivity towards hedaquinium and dequalinium but none was particularly resistant. Both antimicrobial agents inhibited the growth of *Pityrosporum ovale* and *Trichomonas vaginalis*. Both compounds were adsorbed by human or bovine hair on which some remained after repeated washing with water. In cats dequalinium and hedaquinium blocked neuromuscular and ganglionic transmission when injected intravenously in amounts many times greater than the effective dose of suxamethonium chloride. When administered intravenously to mice, hedaquinium in relatively high doses exerted a brief paralysing action.

Dequalinium and hedaquinium are synthetic antimicrobial agents discovered some few years ago in these laboratories[1–5]. Under the names "Dequadin" and "Teoquil" respectively, they are used in various pharmaceutical formulations in the treatment of a variety of non-systemic bacterial and fungal infections. Their antimicrobial activities and general pharmacology have been previously described, and the present paper summarises the results of subsequent investigations.

The possibility of using dequalinium in the form of lozenges for the prevention and treatment of throat and mouth infections made it essential to determine whether dequalinium was antagonised by human saliva. One great advantage of dequalinium over other antibacterial agents used for the treatment of infections of the mouth is that, unlike penicillin for example, it inhibits the growth of *Candida albicans*, and infection with this organism is not a sequel to therapy with lozenges of dequalinium as it frequently is to penicillin therapy. Indeed dequalinium has been used with great success in the treatment of *Candida* infections of the skin, tongue and vagina[6,7]. It was therefore of interest to examine the effect of dequalinium on a number of strains of *C. albicans*. Dequalinium was also shown to inhibit the growth of *Trichomonas vaginalis* and in the form of pessaries it was proposed for the treatment of vaginal infections due to *T. vaginalis* and *C. albicans*[8,9]. It was essential therefore to know the amounts that could be tolerated in the vagina and accordingly

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the local toxicity of dequalinium in the rabbit vagina was tested. The toxicity of dequalinium in the rabbit eye was also investigated, since in the form of eye drops it was proposed for the treatment of infections of the eyes. The adsorption of dequalinium and hedaquinium on hair was tested because these substances have given promising results in the treatment of ringworm in cattle and man[10,11], and preparations of hedaquinium are now widely used for this purpose. The inhibitory action of dequalinium and hedaquinium against *Pityrosporum ovale* was also determined, as this organism is believed by some to be the causative agent of dandruff. Finally, since both substances belong to a group other members of which are muscle relaxants, it was thought desirable to test the neuromuscular blocking activities of the compounds.

Material and Methods

Effect of Antagonists on the Antimicrobial Activity of Dequalinium and Hedaquinium

The following materials were used in these tests: sodium palmitate, sodium oleate, sodium stearate, a commercial liquid soap consisting mainly of sulphonated lorol and the potassium salts of coconut oil fatty acids, Cetrimide, "Teepol", sulphated castor oil, sulphonated lorol, Oxoid "Ionagar" No. 2, Oxoid agar-agar (New Zealand), shredded agar (Korean) and human saliva (collected by expectoration).

Antibacterial tests. The medium consisted of 1 per cent peptone (Difco), 0·5 per cent glucose and 0·5 per cent sodium chloride in distilled water. The antagonists were added to this solution in appropriate amounts and the pH was adjusted to 7·2. Dequalinium and hedaquinium were dissolved in the culture-medium and two-fold serial dilutions were made leaving a final volume of 5 ml. in each tube. The tubes were capped and autoclaved at 10 lb. pressure for 10 minutes. After being allowed to cool, each tube was inoculated with 0·02 ml. of a suspension of *Staph. aureus* CN491 containing approximately 12×10^6 organisms per ml. prepared from a culture grown for 18 hours in dextrose–peptone water. After incubation at 37° the amount of growth was assessed by eye after 5 and 8 days. The results were expressed in terms of minimal inhibitory concentration (M.I.C.).

Antifungal tests. The antagonists were added in suitable amounts to Sabouraud's broth consisting of 1 per cent peptone (Eupeptone No. 2, A. & H.) and 4 per cent glucose in tap water. Double strength aqueous solutions of dequalinium and hedaquinium were mixed with double strength broth and serially diluted two-fold leaving a final volume of 2 ml. in each tube. The tubes were capped and autoclaved at 10 lb. pressure for 10 minutes. A culture of *Candida albicans* 1549 was made by inoculating tubes of Sabouraud's broth and incubating overnight at 37°, and one drop of this culture was used to inoculate the tubes containing the antimicrobial agents and antagonists. The tubes were incubated at 37° for 6 to 8 days and the mean M.I.C. estimated visually.

Effect of dequalinium and hedaquinium on various strains of C. albicans. Cultures of other strains of *C. albicans* were prepared as described for

strain 1549 and the inhibitory activities of dequalinium and hedaquinium were compared with that of nystatin after incubation at 27° for 14 days.

Effect of dequalinium and hedaquinium on Pityrosporum ovale *and* Trichomonas vaginalis. A culture of *P. ovale* was made by inoculating a malt extract agar slope, moistened with (sterile) double cream, and incubated at 37° for 3 days. Dilutions of dequalinium and hedaquinium were prepared in malt extract broth. After autoclaving at 10 lb. pressure for 10 minutes double cream was added aseptically to each tube to give a final concentration of 0·1 per cent. The culture of *P. ovale* was emulsified in saline (20 ml.) and one drop was used to inoculate the tubes. M.I.C. values were determined after incubation at 37° for 7 days.

T. vaginalis was grown in a modification of Feinberg's medium[12] comprising horse serum, liver infusion and Hartley's digest broth at pH 6·8. Dilutions of the antimicrobial agents were made in this medium, but the horse serum fraction was added aseptically to the tubes after autoclaving. A drop of sediment from a 3-day culture was used to inoculate each tube and the M.I.C. values were determined microscopically after 3 days incubation at 37°.

Adsorption of dequalinium and hedaquinium on human and bovine hair. The hair was soaked for 60 minutes in 4 per cent solutions of dequalinium acetate and hedaquinium chloride (expressed as free base), washed by dipping in distilled water and dried for 30 minutes in air. The amount of antifungal agent still remaining in the hair, before and after washing, was roughly determined either by serial dilution of peptone water in which the hair had been soaked for 30 minutes or by serial bisection of individual pieces of hair which were then added to 5 ml. amounts of peptone water. *Staph. aureus* CN491 was used as test organism, the results being expressed as μg. of drug (base) adsorbed per mg. of hair.

Local toxicity of dequalinium and hedaquinium in rabbits. Vaginal. Pessaries containing the drugs were inserted into the vaginas of rabbits on five consecutive days or solutions of the drugs were introduced by means of a catheter. After three weeks the rabbits were killed, the vagina and uteri were examined and specimens of the tissues were submitted for histological examination.

Eyes. Eye drops containing the drugs were applied daily to the right eyes of a number of rabbits and control drops containing no drug were applied to the left eyes. After three weeks' administration the rabbits were killed and the appropriate tissues were examined histologically.

Neuromuscular blocking and ganglionic blocking activities. Cats. Neuromuscular blocking activity was tested by measuring the effects of the drugs when given intravenously on the response of the tibialis muscle to electrical stimulation of its motor nerve, and ganglionic blocking activity by measuring the effect on the nictitating membrane after pre-ganglionic stimulation when the drugs were administered by arterial injection close to the superior cervical ganglion[13].

Mice. Male white mice weighing 12 to 20 g. were injected intravenously with the drugs in saline and the paralysing activity was measured using a rotating drum[14].

RESULTS

Effect of Antagonists on the Inhibitory Action of Dequalinium and Hedaquinium

Soaps and detergents. The results are recorded in Tables I and II.

In concentrations of 0·05 per cent or more, sodium oleate, sodium palmitate and sodium stearate markedly antagonised both drugs, but the

TABLE I

ANTAGONISM BY VARIOUS SOAPS OF DEQUALINIUM AND HEDAQUINIUM AGAINST *Staph. aureus* CN491 IN DEXTROSE PEPTONE WATER

Soap	Percentage of soap in medium	M.I.C. (μg./ml.) at 5 days	
		Dequalinium	Hedaquinium
Sodium oleate	0·05	250	250
	0·1	>224	250
	0·2	>500	>500
Sodium palmitate ..	0·05	250	15·6
	0·1	250	31·2
	0·2	500	62·5
Sodium stearate.. ..	0·05	250	62·5
	0·1	>250	85·5
	0·2	>500	125·0
Liquid soap	0·005	0·312	0·312
	0·01	0·156	0·156
	0·05	0·156	0·625
None	—	0·36	0·125

TABLE II

ANTAGONISM BY VARIOUS DETERGENTS OF DEQUALINIUM AND HEDAQUINIUM AGAINST *Staph. aureus* CN 491 IN DEXTROSE PEPTONE WATER

Detergent	Concentration of detergent in medium	M.I.C. (μg./ml.) at 5 days	
		Dequalinium	Hedaquinium
Teepol	0 per cent	0·312	0·078
	0·0025 ,,	2·5	0·156
	0·005 ,,	2·5	0·625
	0·01 ,,	>10	2·5
Sulphated castor oil ..	0 ,,	0·156	0·078
	0·0025 ,,	5	5
	0·005 ,,	5	5
	0·01 ,,	5	5
Sulphonated lorol ..	0 ,,	0·156	0·078
	0·00125 ,,	0·078	0·312
	0·0025 ,,	0·156	1·25
	0·005 ,,	0·156	0·625
Cetrimide	0 μg/ml.	0·156	0·039
	0·025 ,,	0·312	0·078
	0·05 ,,	0·312	0·156
	0·1 ,,	0·156	0·078

commercial liquid soap did not. Teepol and sulphated castor oil slightly antagonised both dequalinium and hedaquinium, but sulphonated lorol and cetrimide did not.

Agar. The results of experiments with agar as an antagonist of dequalinium and hedaquinium, using *Staph. aureus* as test organism, are

given in Table III. At a concentration of 1·6 per cent comparable to that used in normal agar plates all samples antagonised both dequalinium and hedaquinium. The results of the experiments using *C. albicans* as test organism are shown in Table IV.

TABLE III

ANTAGONISM BY THREE SAMPLES OF AGAR OF DEQUALINIUM AND HEDAQUINIUM AGAINST *Staph. aureus* CN 491 IN DEXTROSE PEPTONE WATER

Agar	Percentage in medium	M.I.C. (μg./ml.) at 5 days	
		Dequalinium	Hedaquinium
Korean shredded agar ..	0·2	0·312	0·625
	0·4	0·442	1·25
	0·8	1·25	1·77
	1·6	2·5	1·77
Ionagar No. 2	0·2	0·625	1·25
	0·4	1·25	1·25
	0·8	2·5	2·5
	1·6	2·5	5·0
Agar-Agar (New Zealand).	0·2	1·25	1·25
	0·4	0·625	2·5
	0·8	2·5	2·5
	1·6	2·5	5·0
None	—	0·156	0·110

TABLE IV

ANTAGONISM BY AGAR OF DEQUALINIUM AND HEDAQUINIUM AGAINST *Candida albicans*

Agar, per cent	M.I.C. (μg./ml.) at 8 days	
	Dequalinium	Hedaquinium
0	1·25	0·31
0·1	2·5	0·62
0·2	5	0·32
0·4	5	0·16
0·8	20	1·25
1·6	40	2·5

The action of dequalinium and hedaquinium on *C. albicans* was antagonised by agar to about the same extent as with *Staph. aureus*.

Saliva. It will be seen from Table V that the addition of 10 per cent of human saliva to the medium slightly reduced the activity of both dequalinium and hedaquinium against *Staph. aureus*.

TABLE V

ANTAGONISM BY HUMAN SALIVA OF DEQUALINIUM AND HEDAQUINIUM AGAINST *Staph. aureus* CN 491 IN DEXTROSE PEPTONE WATER

Percentage of saliva in medium	M.I.C. in (μg./ml.) at 5 days	
	Dequalinium	Hedaquinium
0	0·221	0·055
5	0·625	0·156
10	0·878	0·878

Activity against various strains of C. albicans. In view of the importance of *C. albicans* as a surface pathogen, the activity of dequalinium and hedaquinium against 21 strains of *C. albicans* was compared with that of nystatin. It will be seen from Table VI that the M.I.C. of dequalinium varied between 0·63 and 5·0 μg./ml. and that of hedaquinium between 0·63 and 1·25 μg./ml.

TABLE VI

INHIBITORY ACTIVITIES OF VARIOUS COMPOUNDS AGAINST 21 STRAINS OF *Candida albicans* IN SABOURAUD'S BROTH

Strains	Mean M.I.C. (μg./ml.) at 14 days		
	Nystatin	Dequalinium	Hedaquinium
C. albicans Lab. strain 239	20	1·25	0·89
" " " 1549	20	2·5	0·89
" L.Sch of Hygiene "Vaginal" ..	40	1·25	0·89
" " " " "McGuiness" ..	40	1·25	0·89
" " " " "Mishra" ..	20	1·25	0·89
" " " " "Young" ..	20	1·25	0·89
" " " " Z 247a ..	80	0·63	0·63
" " " " Z 248a ..	80	2·5	0·89
" " " " Z 249	>40	2·5	0·89
" " " " Z 250	20	5·0	1·25
" " " " Z 251	40	1·25	0·89
" " " " Z 252	20	1·25	0·89
" " " " Z 253	40	2·5	0·89
" " " " Z 254	20	1·25	0·89
" " " " Z 255	40	2·5	1·25
" " " " Z 256	40	2·5	0·89
" " " " Z 257	40	2·5	0·89
" " " " Z 258	40	5·0	0·89
" " " " Z 259	40	1·25	1·25
" " " " Z 260	>40	2·5	1·25
" " " " Z 261	>40	2·5	0·89

TABLE VII

INHIBITORY ACTIVITIES OF DEQUALINIUM AND HEDAQUINIUM AGAINST *Pityrosporum ovale* AND *Trichomonas vaginalis*

Substance	Mean M.I.C. (μg./ml.)	
	P. ovale	*T. vaginalis*
Dequalinium chloride	5·1	18
Dequalinium acetate	—	18
Hedaquinium chloride	7·3	25
Diiodohydroxyquinoline ..	—	100

Activity against P. ovale *and* T. vaginalis. It will be seen from Table VII that dequalinium chloride was slightly more active than hedaquinium chloride against both *P. ovale* and *T. vaginalis* and that both were much more effective than diiodohydroxyquinoline against *T. vaginalis.*

Adsorption on human and bovine hair. It will be seen from Table VIII that human and bovine hair when soaked for 60 minutes in 4 per cent solutions of dequalinium and hedaquinium salts adsorbed appreciable quantities from the solutions. Although these amounts were reduced by three successive washings appreciable amounts remained on the hair. Samples of human hair from different sources gave different values, but

bovine hair adsorbed more. When bovine or human hair was stored for two to four weeks before being washed, the amounts of dequalinium and hedaquinium adsorbed on the hair were very little different from the amounts adsorbed by freshly washed hair. The amount adsorbed was, however, proportional to the time of soaking. Little dequalinium was adsorbed from solutions weaker than 4 per cent although detectable amounts of hedaquinium were adsorbed from a 1 per cent solution of the drug.

TABLE VIII

ADSORPTION OF DEQUALINIUM AND HEDAQUINIUM ON HUMAN AND BOVINE HAIR AFTER WASHING FOR 60 MINUTES

	Drug μg./mg. hair	
	Dequalinium 4 per cent	Hedaquinium 4 per cent
Human hair: unwashed	3·2	6·4
1 washing	0·8	3·2
3 washings	0·8	1·6
Bovine hair: unwashed	12	12·8
1 washing	6	1·6
3 washings	3	1·6

Local toxicity in rabbits. Eyes. The application to the eyes of rabbits of solutions containing 0·25, 1 or 4 per cent dequalinium acetate caused some closure or inflammation (Table IX); a 0·06 per cent solution had no detectable effect. Eye drops containing 0·1 per cent or 0·05 per cent of dequalinium chloride also caused no visible reactions.

TABLE IX

OCULAR TOXICITIES OF DEQUALINIUM AND HEDAQUINIUM

Substance	Type of preparation	Conc. per cent	Observations
Dequalinium acetate	Aqueous solution	4·00	Eye closed and did not recover
		1·00	Eye closed but recovered
		0·25	Slight reaction
		0·06	No reaction
Sodium acetate	Aqueous solution	1·90	No reaction
		0·47	"
		0·12	"
		0·03	"
Dequalinium chloride	Eye drops	0·10	No reaction
Dequalinium undecylenate	Oily solution	0·16	Eyelids encrusted but tissues normal
Control	Oil	—	
Dequalinium undecylenate	Aqueous solution	0·03	Eyes normal, and tissues normal
Control	Water	—	
Dequalinium chloride	Urea-sodium acetate solution	0·05	Eyes normal, tissues changed
Control	"	—	Eyes normal, tissues normal
Dequalinium chloride	Urea-glucose solution	0·05	Eyes normal, tissues changed
Control	"	—	Eyes normal, tissues normal
Dequalinium chloride	Sodium sulphatebenzyl alcohol	0·05	Eyes normal, tissues normal
Control	"	—	
Hedaquinium chloride	Aqueous infusion	0·01	Eyes normal
"	"	0·1	Eyes inflamed
Control	Saline solution	—	Eyes normal

Histological examination of eyes that had received 0·05 per cent of dequalinium chloride showed only slight changes or none. Dequalinium undecylenate in a 0·03 per cent aqueous solution had no adverse effect. A 0·1 per cent solution of hedaquinium chloride caused some inflammation of the eye, but a 0·01 per cent solution had no adverse effect.

TABLE X

VAGINAL TOXICITIES OF DEQUALINIUM AND HEDAQUINIUM

Substance	Type of preparation	Conc. per cent	Observations
Dequalinium acetate ..	Aqueous solution ..	1·00	Refused injections } vagina thickened and vascularised
		0·20	" " }
		0·04	No refusals
Sodium acetate	Aqueous solution ..	0·47	Normal
Dequalinium acetate ..	Aqueous solution ..	0·10	No refusals, vaginas inflamed and granulated
Dequalinium chloride ..	Pessary	0·10 }	Pessaries ejected within 5 minutes
		0·03 }	
		0·01 }	
Control	Pessary	—	Pessaries retained
Dequalinium chloride ..	Cones	0·2 mg./cone	Cones retained but only ¼ in. into vaginas
Dequalinium chloride ..	Urea solution, 50 per cent	0·02 }	No refusals, vaginas inflamed
		0·1 }	
Control	Urea solution, 50 per cent	—	Normal
Hedaquinium chloride ..	Aqueous infusion ..	0·01	Vaginas normal
"	" "	0·1	Vaginas vascularised
Control	Saline solution	—	Vaginas normal

Vaginal. Solutions of dequalinium salts were infused intravaginally in rabbits on successive days. The results are summarised in Table X. Solutions containing 0·2 per cent or 1 per cent of dequalinium acetate caused obvious discomfort, but solutions containing 0·1 per cent or less of dequalinium acetate and chloride were tolerated. On histological examination, however, inflammation was observed in all vaginas receiving a 0·02 per cent solution of dequalinium chloride or a 0·04 per cent solution of dequalinium acetate. Pessaries containing 0·01, 0·03 or 0·1 per cent of dequalinium chloride were ejected whereas control pessaries containing no drug were retained by the rabbits. Infusions of hedaquinium chloride did not cause visible reactions in the vagina at a concentration of 0·1 per cent but on histological examination some inflammation was noted. A 0·01 per cent solution did not cause inflammation.

Neuromuscular block. Dequalinium chloride administered intravenously to two cats caused some diminution in the response of the tibialis to intermittent electrical stimulation of its motor nerve, 1 mg./kg. giving a 78 per cent reduction in tension. Doses of 2 and 3 mg./kg. of hedaquinium methosulphate gave a 67 and a 99 per cent reduction respectively. In a direct comparison with suxamethonium chloride, hedaquinium methosulphate was found to have 1/200th the potency and about twice the duration of activity. When given by arterial injection close to the superior cervical ganglion, hedaquinium methosulphate at 2 mg./kg. produced a 50 to 90 per cent block in the contraction of the

nictitating membrane; recovery was complete. Hedaquinium methosulphate injected intravenously produced paralysis in mice, causing them to fall off the drum, the value of ED50 being 2·2 ± 0·3 mg./kg.

Discussion

The marked antagonistic action of soaps on both dequalinium and hedaquinium shows that soaps must not be used in the formulation of pharmaceutical preparations containing these antimicrobial substances, and that soaps must not be used in conjunction with such preparations. Where cleansing of skin, or hair is necessary, a detergent that does not reduce the antibacterial or antifungal activity of dequalinium or hedaquinium should be chosen.

The inhibitory effect of agar on the two substances explained some serious discrepancies observed when preparations were assayed by the serial dilution method and by the agar plate method. We recommend that the latter method should not be used, as low results will be obtained. Even with 10 per cent of human saliva in the medium dequalinium was found to be still effective against *Staph. aureus* in a concentration of one part per million.

Dequalinium has been found to be successful in the treatment of local infections with *Candida albicans* both in the mouth and in the vagina. It had been suggested that some strains might be more resistant than strain 1549 with which the initial investigations were carried out. The results reported here, however, show that although there is in fact a variation in the sensitivity of different strains, even the most resistant is inhibited by 5 parts per million. The observations with *Trichomonas vaginalis* showed that dequalinium chloride and acetate are more effective inhibitors of this organism in our tests than the widely-used diiodohydroxyquinoline.

Preparations with hedaquinium chloride as the active agent are being used with success in the treatment of fungal infections in man and in farm animals[10,11]. It was of interest to determine whether hair treated with hedaquinium would retain any of the antifungal agent after washing.

The experiments described above showed that solutions containing 0·1 per cent of dequalinium chloride had no irritant action on the eyes of rabbits. Accordingly the concentration of dequalinium chloride for eye drops was fixed at 0·1 per cent, clinical tests confirming that at this dilution the solution did not cause irritation in the human eye. The rabbit's vagina appeared to be more sensitive than the eye to dequalinium and the lowest concentration used in the animal tests and found to be only slightly irritant, namely 0·01 per cent, was selected in making pessaries for trials in women. Pessaries containing 0·01 per cent of dequalinium chloride were in fact well tolerated and gave satisfactory results in *C. albicans* and *T. vaginalis* infections[8,9].

The experiments on the neuromuscular blocking activities of the two antimicrobial agents showed that these were active by intravenous injection only when given in concentrations greatly in excess of the effective levels of a potent muscle relaxant such as suxamethonium. Dequalinium

and hedaquinium are recommended only for oral or topical therapy, their systemic toxicities precluding their use by parenteral administration. In an unpublished investigation of the metabolism of dequalinium it was observed that when large doses were given by mouth to rats none could be detected in the blood, all of the dose apparently being excreted in the faeces. It seems unlikely therefore that dequalinium will produce neuromuscular block when given orally.

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REFERENCES

1. Collier, Potter and Taylor, *Brit. J. Pharmacol.*, 1953, **8**, 34.
2. Collier, Potter and Taylor, *ibid.*, 1955, **10**, 343.
3. Babbs, Collier, Austin, Potter and Taylor, *J. Pharm. Pharmacol.*, 1956, **8**, 110.
4. Collier and Grimshaw, *Brit. J. Pharmacol.*, 1958, **13**, 231.
5. Austin, Potter and Taylor, *J. chem. Soc.*, 1958, 1489.
6. Coles, Grubb, Mathuranagagam and Wilkinson, *Brit. med. J.*, 1958, **2**, 1014.
7. Stockdale and Banks, *Practitioner*, 1959, **182**, 215.
8. Roddie, *Malayan med. J.*, 1958, **13**, 171.
9. Levinson, *Practitioner*, 1959, **183**, 195.
10. Colin-Jones, *Med. Press*, 1958, 710.
11. Gold and Jones, *Brit. vet. J.*, 1958, **114**, 377.
12. Feinberg, *Nature, Lond.*, 1953, **171**, 1165.
13. Paton and Zaimis, *Brit. J. Pharmacol.*, 1949, **4**, 381.
14. Collier, Hall and Fieller, *Analyst*, 1949, **74**, 592.

THE QUANTITATIVE RESPONSE OF THE OXYGEN CONSUMPTION AND WEIGHT OF GUINEA PIGS TO SOME METABOLIC STIMULANTS. WITH A NOTE ON (±)-5-IODOTRYPTOPHAN

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Regression coefficients for dose and oxygen consumption have been derived for 2,4-dinitrophenol (2,4-DNP), 4,6-dinitro-*o*-cresol (DNC), 2,6-dinitrophenol (2,6-DNP), and (−)-thyroxine. (±)-5-Iodotryptophan has no significant effect on the oxygen consumption or weight of guinea pigs.

A modified form of equipment for the measurement of oxygen consumption of small animals has been described in a previous communication[1]. It can be used to demonstrate a significant relation between increase in oxygen consumption and dose of various metabolic stimulants[1,2]. When log per cent increase in oxygen consumption was plotted against log dose for 2,4-dinitrophenol (2,4-DNP), and 4,6-dinitro-*o*-cresol (DNC), the relation appeared to be linear. A similar response was noted with (−)-thyroxine.

Because several investigators[3–5] have measured metabolic activities by plotting increase in oxygen consumption against time it seemed reasonable to investigate the possibility of extracting a linear relationship from the response. Included in this communication, therefore, are regression coefficients calculated on previous data[1,2] and on fresh experiments, including assays on 2,6-DNP and (−)-thyroxine. Special attention has been paid to 2,6-DNP because not only did it cause moderate increases in the oxygen consumption of guinea pigs, but it also caused a hypothermia in rats, particularly at high dose levels[2].

In addition a note has been included on a new synthetic amino acid, (±)-5-iodotryptophan[6], to report experiments that have demonstrated its inability to affect the oxygen consumption or body weight of guinea pigs. Although this amino acid was prepared for another biological investigation, it was considered desirable to test its possible effects on the overall basal metabolism of guinea pigs particularly as it contained an aromatic nucleus containing iodine.

Methods

Animals. When possible equal numbers of male and female guinea pigs weighing 250 to 350 g. were used for all dose levels of all substances.

Number of doses. Since earlier studies[2] confirmed that maximum levels of the dinitrophenols occurred 1 to 2 hours after administration, oxygen consumption values were obtained 1·25 to 1·5 hours after *single* intraperitoneal doses.

A different procedure was adopted for thyroxine, and a preliminary experiment demonstrated that within dose limits the hormone exercised

a cumulative action after several daily doses. In consequence of this *four* daily doses of thyroxine were always given before the final oxygen consumption was measured 24 hours after the last dose.

First and last values for oxygen consumption *were calculated on the weight of the animal at the time of the measurement.* This is particularly important when thyroxine is used for several days since the weight loss may be 20 per cent or more. When dinitrophenols are assayed a single weight is sufficient because these substances reach their maximum effects on oxygen consumption very rapidly.

All substances were administered as aqueous solutions of their sodium salts.

Plan of Dosing

Substance	Number of animals	Number of animals per dose	Dose mg./kg.
2,4-*DNP*			
Scheme A (*a*)	18	3	5, 10, 20, 25, 30, 35.
Scheme B (*a*)	36	6	5, 10, 20, 25, 30, 35.
DNC	24	6	5, 10, 15, 20.
2,6-*DNP*	24	6	5, 10, 20, 30.
(−)-*thyroxine*	30	5	0·125, 0·25, 0·50, 1·0, 2·0(*b*).
(±)-5-*iodotryptophan*	18	3	1·4 × 2 mg./kg. thyroxine. 2·4 × 2 mg./kg. thyroxine plus 4 × 2 mg./kg. (±)-5-iodotryptophan. 3·4 × 2 mg./kg.(±)-5-iodotryptophan.

Note.—(*a*) See Harvey[1]. (*b*) See note on Table III.

Results

The cumulative effects of four daily doses of 1 mg. of thyroxine per kg. on the oxygen consumption and weights of guinea pigs are shown in Figure 1. Regression coefficients and equations for 2,4-DNP and DNC are shown in Table I, and comparison of their residual variances in Table II. Equations have not been derived for any of the male or female regression

TABLE I

Regression coefficients and equations for 2,4-DNP and DNC

Type of measurement	*n*	$b \pm$S.D.	P	Equation
2,4-*DNP, Scheme A.*				
All doses, all animals	18	$+1{\cdot}20 \pm 0{\cdot}37$	$>0{\cdot}001 < 0{\cdot}01$	$y = 0{\cdot}123 + 1{\cdot}20x$
All doses, males	9	$+0{\cdot}84 \pm 0{\cdot}12$	$<0{\cdot}001$	(*a*)
All doses female	9	$+1{\cdot}89 \pm 0{\cdot}79$	$>0{\cdot}01 < 0{\cdot}05$	(*a*)
Group 1	6	$+1{\cdot}65 \pm 0{\cdot}91$	$>0{\cdot}01 < 0{\cdot}05$	(*a*)
Group 2	6	$+0{\cdot}57 \pm 0{\cdot}74$	N.S.	—
Group 3	6	$+1{\cdot}32 \pm 0{\cdot}06$	$<0{\cdot}001$	(*a*)
2,4-*DNP, Scheme B*				
All doses, all animals	35	(*b*) $+1{\cdot}12 \pm 0{\cdot}17$	$<0{\cdot}001$	$y = 0{\cdot}253 + 1{\cdot}12x$
5, 10, 20 mg./kg. (*c*)	17	(*b*) $+0{\cdot}99 \pm 0{\cdot}23$	$\sim 0{\cdot}01$	$y = 0{\cdot}371 + 0{\cdot}99x$
10, 20, 35 mg./kg. (*c*)	18	$+1{\cdot}65 \pm 0{\cdot}25$	$<0{\cdot}001$	$y = 1{\cdot}75x - 0{\cdot}514$
25, 30, 35 mg./kg. (*c*)	18	$+3{\cdot}45 \pm 0{\cdot}85$	$<0{\cdot}001$	$y = 3{\cdot}45x - 3{\cdot}167$
All doses, males	17	(*b*) $+1{\cdot}48 \pm 0{\cdot}24$	$<0{\cdot}001$	(*a*)
All doses, females	18	$+0{\cdot}93 \pm 0{\cdot}23$	$<0{\cdot}001$	(*a*)
Group	6	$+1{\cdot}48 \pm 0{\cdot}16$	$<0{\cdot}001$	$y = 1{\cdot}48x - 0{\cdot}216$
Mean of six values per dose	6	$+1{\cdot}10 \pm 0{\cdot}17$	$>0{\cdot}01 > 0{\cdot}001$	$y = 0{\cdot}324 + 1{\cdot}10x$
DNC				
Group	4	$2{\cdot}23 \pm 0{\cdot}34$	$>0{\cdot}01 < 0{\cdot}05$	$y = 2{\cdot}33x - 1{\cdot}282$

Notes—
(*a*) Variation between four sex values or small group values too great for satisfactory equations.
(*b*) One negative value disregarded.
(c) Overall regression for these three values $1{\cdot}42 \pm 0{\cdot}19$ (see Table II) giving equation: $y = 1{\cdot}42x - 0{\cdot}119$.

values because of significant differences, or for the three small group values in Scheme A. Some of the significant regressions have been plotted graphically on Figure 2.

Because of the apparently anomalous behaviour of 2,6-DNP in causing limited increases in oxygen consumption and also a significant hypothermia[2] a fuller investigation was made on this substance. The results of this experiment are shown in Figure 3. From this it is seen that the mean per cent increases in oxygen consumption per group were, at 5 mg./kg. + 25·7 per cent, at 10 mg./kg. + 28·3 per cent, at 20 mg./kg. + 14·1 per cent and at 30 mg./kg. − 23·1 per cent.

One large increase (76·7 per cent) made the last average disproportionately great. Omitting this value the mean would have been 10·1 per cent. These results suggested two phases of activity, first, an initial stimulation, significantly positive, but of short duration, and secondly, a negative response reducing the first initial increase to within normal limits. This trend was in accordance with the observations on the effects of this dinitrophenol on body temperature.

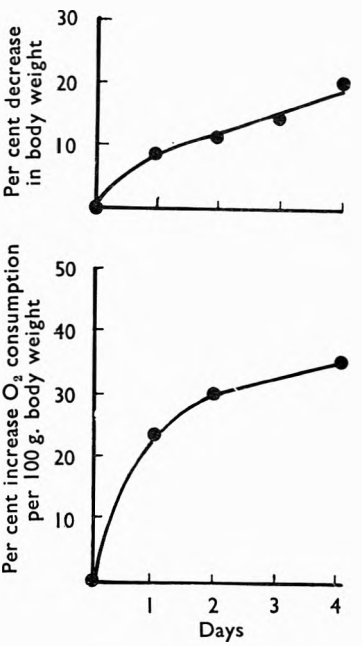

FIG. 1. Increasing oxygen consumption and decreasing weight of guinea pigs. Mean value of 3 male and 3 female animals each given 4 daily doses of 1 mg/kg. Na-(−)-thyroxine.

A regression coefficient was calculated for all doses except the first.

Dose levels	n	$b \pm$S. D.	P
10, 20, 30 mg./kg.	17 (*a*)	−0·87 ± 0·52	N.S. at 0·20 (*b*)

(*a*) One reading lost.

(*b*) For $n_1 = 1$, $n_2 = 17$, F = 1·8 at P = 0·20.

TABLE II

COMPARISON OF REGRESSIONS BY RESIDUAL VARIANCES 2,4-DNP

Type of measurement	D.F.	Variance, ratios and significance
2,4-DNP		
All doses, all animals		
Scheme A	16	0·219 } 2·54 Nearly significance at P = 0·01
Scheme B	33	0·086 }
5, 10, 20 mg./kg. ..	15	0·1123 } 1·9 N.S. } 2·6, P < 0·05
10, 20, 30 mg./kg. ..	16	0·0591 } 1·4 N.S. }
25, 30, 35 mg./kg. ..	16	0·0432 }

The overall regression for the last three regressions is 1·42 ± 0·19 with a residual variance of 0·070. This does not vary significantly from the All doses all animals or Group Regressions variances of Scheme B. (P < 0·01).

Regression coefficients and equations for relations between dose of thyroxine, oxygen consumption and weight are given in Table III, and the residual variances in Table IV. The relation between oxygen consumption and dose is also illustrated in Figure 4. It is noted that the

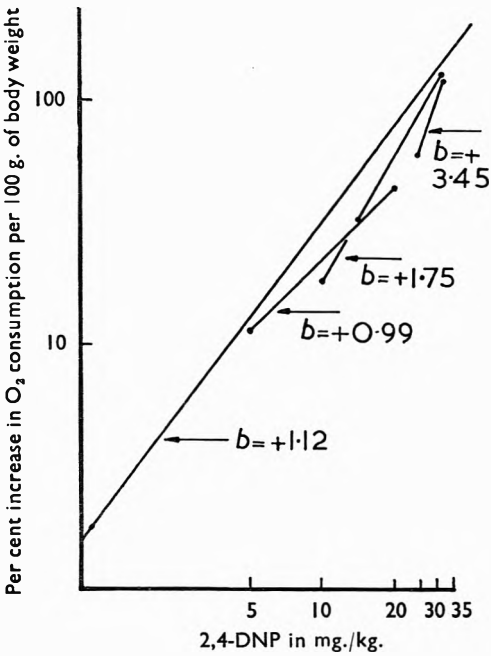

FIG. 2. Various regressions of oxygen consumption and dose of DNP in the guinea pig. (Scheme B).

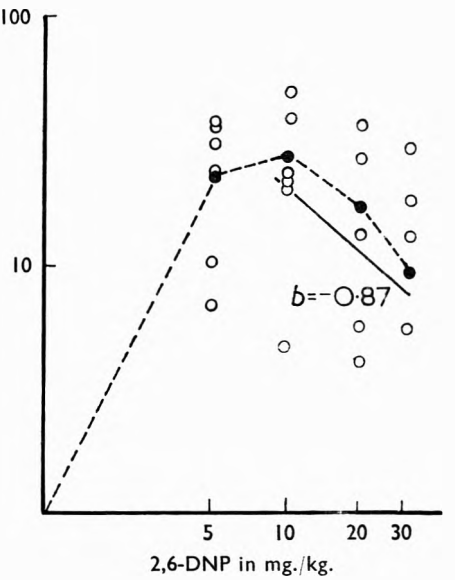

FIG. 3. Partial stimulation of oxygen consumption of guinea pigs by 2:6-DNP. ●---● Mean values.

dose: weight response is more significant than the oxygen consumption dose relation.

TABLE III

REGRESSION COEFFICIENTS AND EQUATIONS FOR THYROXINE

Type of measurement	*n*	$b \pm$S.D.	P	Equation
Thyroxine—oxygen consumption (*c*)				
All doses, all animals	22	(*a*) $+0{\cdot}59 \pm 0{\cdot}26$	$>0{\cdot}01 < 0{\cdot}05$	$y = 0{\cdot}022 - 0{\cdot}59x$
All doses, males	15	$+0{\cdot}25 \pm 0{\cdot}13$	$>0{\cdot}05 < 0{\cdot}20$	(*b*)
All doses, females	7	(*a*) $+1{\cdot}66 \pm 0{\cdot}61$	$>0{\cdot}02 < 0{\cdot}05$	(*b*)
Group	4	$+0{\cdot}63 \pm 0{\cdot}19$	$>0{\cdot}05 < 0{\cdot}20$	$y = 063x - 0{\cdot}167$
Mean of six values per dose	4	$+0{\cdot}60 \pm 0{\cdot}20$	$>0{\cdot}05 < 0{\cdot}20$	$y = 0{\cdot}073 + 0{\cdot}60x$
Thyroxine—body weight (*c*)				
All doses, all animals	24	$+0{\cdot}34 \pm 0{\cdot}08$	$<0{\cdot}001$	$y = 0{\cdot}240 + 0{\cdot}34x$
All doses, males	15	$+0{\cdot}29 \pm 0{\cdot}01$	$>0{\cdot}01 < 0{\cdot}05$	$y = 0{\cdot}77 + 0{\cdot}29x$
All doses, females	9	$+0{\cdot}39 \pm 0{\cdot}04$	$<0{\cdot}001$	$y = 0{\cdot}092 + 0{\cdot}39x$
Group	4	$+0{\cdot}36 \pm 0{\cdot}11$	$>0{\cdot}05 < 0{\cdot}20$	$y = 0{\cdot}192 + 0{\cdot}36x$

Notes.

(*a*) Two negative values disregarded.
(*b*) Regressions not significant enough for good equations.
(*c*) Regressions calculated on 4 dose levels only (0·125, 0·25, 0·5, 1·0 mg./kg.) because 2 mg./kg. did not cause any increases in oxygen consumption greater than those caused by 1 mg./kg.

To investigate further the scatter of individual values within dose groups the coefficients of variation for the mean oxygen consumption for each dose were determined for 2,4-DNP (Scheme B), and for (—)-thyroxine. These are shown in Table V.

The results of the experiments on 5-iodotryptophan are given in Table VI.

Discussion

Three general observations may be made from the results shown in Table V. First, the response is more homogeneous at higher than at lower dose levels of 2,4-DNP, in fact, there is a significant regression between the coefficient of variation and dose (b — 1·05 ± 0·15, P < 0·01 > 0·001). Secondly, there is a similar *trend*, but not very significant, in the case of thyroxine (b = — 29·1, P > 0·20). Thirdly, the weight changes do not vary significantly (11·9 ± 3·0).

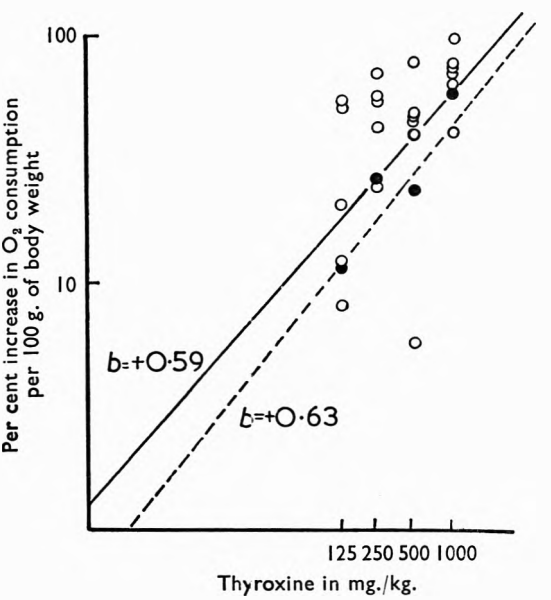

FIG. 4. Regression of oxygen consumption and doses of Na(—)-thyroxine in the guinea pig. All doses all animals, ○——○. Group, ●- - -●.

From the experiments with 5-iodotryptophan (Table VI) it is clear that this amino acid has no significant effect on increasing the oxygen consumption or lowering the body weight of guinea pigs. Neither does it appear to possess anti-thyroxine action, since it does not diminish the response of guinea pigs given simultaneous doses of thyroxine.

A completely satisfactory method for the assay of metabolic stimulants, particularly thyroxine, has not yet been evolved, but these results show sufficient promise to warrant further investigation. The measurement of oxygen consumption in large numbers of small animals presents many technical difficulties, especially that of achieving thoroughly sound "basal" conditions, but it is considered that an approach to the problem along the lines developed in the present studies should eliminate many of these obstacles.

It has proved possible in these experiments to derive a significant relation between dose and oxygen consumption, particularly when synthetic stimulants are employed. Similar but less significant relations

TABLE IV

COMPARISON OF REGRESSIONS BY RESIDUAL VARIANCES—THYROXINE

Type of measurement	D.F.	Variance, ratios and significance
Thyroxine—oxygen consumption		
All doses, all animals	20	0·169
All doses, male	13	a
All doses, female	5	a
Group	4	0·0352
Mean six values per dose	4	0·0359
		1·0 N.S.
		9·0 N.S.
Thyroxine—weight		
All doses, all animals	22	0·0193
All doses, male	13	0·018
Al doses, female	7	0·0016
Group	4	0·0061
		1·1 N.S.
		11·2 P 0·01
		12·0 P 0·01
		3·1 N.S.

a. See data in Table III; as no equation derived, no significance or ratios calculated.

TABLE V

SCATTER OF OXYGEN CONSUMPTION AND BODY WEIGHT VALUES FOR DIFFERENT DOSE LEVELS OF 2,4-DNP AND THYROXINE

Coefficient of variation

2,4-DNP		(−)-Thyroxine		
Dose mg./kg.	Oxygen consumption	Dose µg./kg.	Oxygen consumption	Body weight
5	34·5	125	42·7	11·9
10	28·8	250	10·5	16·1
20	18·4	500	26·1	9·2
25	17·7	1000	6·6	10·4
30	7·9			
35	6·6			

TABLE VI

(±)-5-IODOTRYPTOPHAN ON THE OXYGEN CONSUMPTION AND BODY WEIGHT OF THREE MALE AND THREE FEMALE GUINEA PIGS

Na-(−)-thyroxine only, 4 daily doses, 2 mg./kg.		Na-(−)-thyroxine, and (±)-5-iodotryptophan 4 daily doses, 2 mg./kg. each substance		(±)-5-iodotryptophan 4 daily doses, 2 mg./kg.			
					Oxygen consumption		
					Actual ml./100 g. min.		
Weight per cent change	Oxygen per cent change	Weight per cent change	Oxygen per cent change	Weight per cent change	Before	After	Per cent change
−14·7	+44·5	−13·1	+4·3	+2·7	2·17	2·04	−5·9
−14·2	+47·5	−24·5	+48·8	−2·1	1·90	2·25	+18·4
−8·3	+27·0	−11·0	+53·5	0·0	1·75	2·05	+17·1
−20·5	+22·5	−14·8	+82·2	−2·6	1·55	1·62	+4·5
−15·2	+30·5	−9·9	+48·7	+1·0	2·19	1·70	−22·3
−8·4	+39·0	11·7	+27·7	+1·5	1·96	1·82	−7·1
Averages							
−13·6	+35·1	−14·2	+44·2	0·1	1·92	1·91	+0·8

Tests on Data in Table VI

Since there are n observations there are n-1 (5) degrees of freedom and for P=0·05 t should be 2·57.

1. *Comparison between thyroxine and thyroxine plus 5-iodotryptophan treatments*
   (*a*) Between weight changes: t = 0·25, N.S.
   (*b*) Between changes in oxygen consumption t = 2·02, N.S.
2. *Comparison between treatments—before and after 5-iodotryptophan only*
   (*a*) Between individual values, oxygen consumption t = 0·03 N.S.
   (*b*) Between weights, differences very small, no t test applied.

can be derived for (—)-thyroxine. Extrapolation of the regressions to the intersection of the x and y axes (Figs. 2 and 4) suggests that over a wide dose range the linear relation holds good. For, although the regressions do not pass exactly through the intersections, the variations are small—usually about 5 per cent.

The minimum number of animals necessary for a satisfactory regression appears to lie between 20 and 30 with at least 4 dose levels. Small numbers (for example, Scheme A, 2,4-DNP) are likely to give variable results. Evidence in support of these suggestions may be obtained by examining the regressions resulting from random selection of 1,2 . . . 5 increased oxygen consumption values for each dose level of 2,4-DNP in Scheme B. Thus:

Number of animals selected at random from each group	Total number of animals	$b \pm$ S.D.	Coefficient of variation
1	6	$+1{\cdot}49 \pm 0{\cdot}40$	26·9
2	11	$+1{\cdot}14 \pm 0{\cdot}24$	21·1
3	18	$+1{\cdot}19 \pm 0{\cdot}25$	20·9
4	24	$+1{\cdot}15 \pm 0{\cdot}25$	21·6
5	30	$+0{\cdot}96 \pm 0{\cdot}20$	20·7
	Average	$+1{\cdot}17 \pm 0{\cdot}27$	
6 (*vide supra*)	35	$+1{\cdot}12 \pm 0{\cdot}17$	15·1

The average regression from 1 to 5 random selections is seen to be only 5 per cent greater than that obtained from the all doses, all animals regression; also the coefficient of variation is greater with a smaller than with a larger number of animals.

It appears that equal numbers of male and female animals are essential if sufficient numbers of either sex are not available. With 2,4-DNP the variations between the four sex regressions from Schemes A and B are highly significant, and therefore mean values for males and females, namely, 1·41 and 1·16 cannot be used in calculating and overall regression.

The selection of appropriate dose levels for 2,4-DNP type compounds presents fewer problems and the overall regression calculated on the three dose groups in Scheme B does not vary significantly from all the doses, all animals and Group regressions. On the whole, Group responses are reliable (see Maclagan and Sheahan[4]) and they have the advantages of speed, simplicity and probably the elimination of individual variations[1].

The experiments on thyroxine show that the weight decrease : dose relation is better than the oxygen consumption : dose relation. This is in general agreement with the observations of Reinecke and Turner[7], and it is difficult to appreciate why this simple technique has not received further attention (see Burn[3] commenting on Kreitmair[9]).

One other feature that requires further investigation is the observation that increasing doses of thyroxine often cause a reduction in response. Maclagan and Shehan[4] report steady increases with doses of thyroxine above 2 mg./kg. on mice, but in my experiments the maximal dose for guinea pigs is 4 daily doses of 1 mg./kg. A fuller study is desirable to provide more basic information for the design of an adequate assay technique.

Acknowledgements. I wish to thank Mr. David Chessell and Miss Angela Hunt for valuable technical assistance, and Dr. J. D. Biggers for many helpful suggestions. I also wish to thank the Medical Research Council for a grant.

References

1. Harvey, *J. Pharm. Pharmacol.*, 1953, **10**, 483.
2. Harvey, *ibid.*, 1959, **11**, 462.
3. Gaddum, *J. Physiol.*, 1930, **68**, 383.
4. Maclagan and Sheahan, *J. Endocrinol.*, 1950, **8**, 456.
5. Holtkamp, Ochs, Pfeiffer and Heming, *Endocrinol.*, 1955, **56**, 93.
6. Harvey, *J. chem. Soc.*, 1958, 3760.
7. Reinecke and Turner, *Res. Bull. Mo. agric. Sta.*, No. 355, 1942.
8. Burn, Biological Standardisation, 2nd Edn., Oxford Medical Publications, 1950.
9. Kreitmar, *Z. exp. Med.*, 1928, **61**, 202.

THE WATER-SOLUBLE CARBOHYDRATES OF *PAPAVER SOMNIFERUM* L.

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From an aqueous extract of opium poppy capsules of Yugoslavian origin, the following carbohydrates were isolated: glucose, fructose, sucrose, sedoheptulose, mannoheptulose, plus a complex polysaccharide. The polysaccharide was isolated in two different ways, by means of an ion exchange resin and by precipitation with ethanol. The first-mentioned procedure appeared to give the most genuine product, as the substance thus obtained formed a gel when sulphuric acid was added to an aqueous solution. Enzymatic hydrolysis with a pectase preparation liberated arabinose. The product isolated by ethanol precipitation gave no arabinose upon treatment with the enzyme preparation. By hydrolysis with acid, both products yielded the same monosaccharides, arabinose, xylose, rhamnose, glucose, galactose, uronic acid, and an unidentified component.

During the last two to three decades, increasing amounts of morphine have been manufactured directly from opium poppy capsules[1]. As the chemical composition of the poppy capsules is different from that of opium, the morphine manufacturers have to face numerous new problems when changing from opium to opium poppy capsules as their starting material.

In the manufacture of morphine and other opium alkaloids from the capsules, the starting material is usually extracted with water or with various aqueous solutions. The main difficulties in working with such extracts are caused by the huge amount of pectic substances present, but practically nothing has been done to investigate the physical properties and the chemical composition of these substances. It, therefore, seemed important from a scientific, as well as a practical point of view, to undertake a study of the water-soluble carbohydrates of the opium poppy capsules. Dried poppy capsules without seeds were used for this work. The material was obtained from Yugoslavia from a white-seeded poppy variety†.

Experimental

Free Sugars

Eighty g. of poppy capsules was ground to a coarse powder and macerated for 16 hours with 750 ml. of 80 per cent ethanol. The extract was filtered and the ethanol removed under reduced pressure (25 mm. Hg).

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† The authors are indebted to Weiders Farmasöytiske A/S, Oslo, for supplying the necessary material.

The residue, amounting to 75 ml. of a brown, turbid mixture, was centrifuged and purified by precipitation with 10 ml. of basic lead acetate solution, followed by filtration and de-ionisation of the filtrate with Amberlite IR-120 and Amberlite IR-4B.

The partly purified solution was evaporated under reduced pressure to a syrupy liquid from which the free sugars were extracted with 1 to 2 times its volume of hot ethanol. The filtered solution gave positive tests for free sugars[2]. Also, reactions indicating the presence of pentoses and heptoses, such as Rosenthalers'[3], Bial's[4] and Thomas'[5] reactions, were positive.

The sugar solution was chromatographed by the descending method on Whatman No. 1 filter paper using pure sugars as reference substances. In this way, glucose, fructose, sucrose, mannoheptulose and sedoheptulose were detected. A solvent mixture consisting of ethyl acetate, acetic acid and water (3:1:3) was used for separation of the three ketoses, and the spots were developed with a ketoheptulose reagent[6,7].

Separation and identification of glucose was accomplished by means of an ethyl acetate-pyridine-water mixture (2:1:2), using aniline-phthalate[8] as a spray reagent. No galactose could be detected in the mixture.

To confirm the identification of the heptoses, sucrose and free hexoses were removed by fermentation as follows. The alcoholic solution of the sugar mixture (25 ml.) was evaporated to dryness on a steam bath and the residue dissolved in 10 ml. of water. Bakers' yeast was added, and the mixture allowed to ferment for 6 hours at 35°, after which it was filtered and the filtrate evaporated to dryness. The residue was extracted with 50 per cent ethanol, the extract filtered and chromatographed on filter paper as described above. Treatment with the ketoheptulose reagent[6,7] produced two distinct spots, corresponding to mannoheptulose and sedoheptulose, plus one very faint spot of sucrose.

Water-soluble Polysaccharides

Isolation by adsorption on an ion exchange resin. In the course of a project aimed at developing a satisfactory assay procedure for morphine in poppy capsules, the results of which will be published elsewhere, the powdered capsule material was shaken with an aqueous suspension of a cationic exchange resin of the sulphonated polystyrene type (Dowex 50-X_2, (H^+)). It was found that not only the alkaloids, but also the pectic polysaccharides were adsorbed on the resin. Because these polysaccharides caused difficulties during the elution and purification of morphine, they were removed by selective elution with a dilute aqueous pyridine solution. This eluate, which was light yellow in colour and rather viscous, was used as a source of polysaccharides. The solution was concentrated under reduced pressure to a small volume and precipitated with ethanol. The polysaccharides were filtered off and purified by repeated solution in water and precipitation with ethanol.

Larger amounts of the polysaccharides were isolated in the following way. A mixture of 400 g. of Dowex 50-X_2 (H^+), 100 g. of poppy capsules

(coarse powder) and 3 l. of water was shaken mechanically for 3 hours. It was then set aside for a short while to allow the heavy ion exchange resin to settle to the bottom, whereupon the supernatant aqueous suspension of the extracted capsule powder was poured off. By means of a few washings with water followed by careful decantation, most of the capsule powder could be removed. Finally, the ion exchange resin was transferred to a large chromatographic column fitted with a stopcock, and the polysaccharides were eluted with a 0·5 per cent solution of pyridine in water. The eluate was concentrated under reduced pressure and the polysaccharides precipitated with ethanol. The precipitate was purified as described above and dried in a vacuum desiccator. Yield: 0·70 g. of a yellowish-brown, gum-like product.

An aqueous solution of the polysaccharides (1 : 35) was brown, viscous and turbid. It had a pH of 3·1. The equivalent weight, as determined by titration with alkali, was 501. Qualitative tests for sugars and uronic acids were positive. A gel was formed when sulphuric acid was added to the aqueous solution.

The polysaccharides were hydrolysed by heating 0·70 g. of the substance with 70 ml. of N sulphuric acid in a closed container at 100° for 7 hours. During hydrolysis the solution lost its viscosity, and a grayish-brown precipitate separated out. As this precipitate gave negative tests for sugars, it was filtered off and the filtrate neutralised with 45 g. of barium carbonate. The precipitate of barium sulphate was removed by filtration and washed with water, and the combined filtrate and washings evaporated to 20 ml. under reduced pressure. Addition of ethanol produced a precipitate, separating the hydrolysate into an ethanol-soluble and an ethanol-insoluble part.

The ethanol-soluble part of the hydrolysate was concentrated and analysed by means of paper chromatography as described above. The following monosaccharides were identified: arabinose, xylose, rhamnose, glucose, galactose, traces of a uronic acid, plus an unidentified sugar component with a high R_F value.

The carbohydrates of the sugar mixture were also separated and isolated on a column of Whatman cellulose powder[8]. The isolated sugars were converted into osazones and identified by means of their micro melting points (Kofler). Authentic samples of the corresponding osazones were used as reference substances. The results are shown in Table I.

TABLE I

MELTING POINTS OF OSAZONES OF ISOLATED AND OF AUTHENTIC CARBOHYDRATES

Name of compound	Melting point of component from hydrolysis °C	Melting point of authentic sample °C
Arabinosazone	155	155
Xylosazone	167	166·5
Rhamnosazone	185–187	187
Glucosazone	202	205·5–207
Galactosazone	170–187	190–193

The column chromatography technique also revealed a compound which gave the reactions of a uronic acid. The compound is probably galacturonic acid, as no lactone could be detected by means of paper chromatography, and the oxidation product yielded a substance that melted at 215 to 217° (Kofler). Under corresponding conditions an authentic sample of mucic acid melted at 215 to 217°.

The ethanol-insoluble part of the hydrolysate gave a strong test for uronic acids. It was purified by repeated solution in water and precipitated with ethanol. It was then dissolved in 2N sulphuric acid and heated at 100° for 16 hours. The reaction mixture was filtered, neutralised with barium carbonate and filtered again. The Ba^{++} ions were removed from the filtrate by means of a cationic exchange resin (Amberlite IR-120). The solution obtained in this way was evaporated to dryness, the residue was treated with ethanol and the ethanol-soluble part analysed by means of paper chromatography, which revealed the presence of a uronic acid.

Enzymatic degradation of the polysaccharides was as follows. One hundred mg. of the polysaccharide was dissolved in 10 ml. of McIlvaine's buffer[9] of pH 5·0. Two mg. of pectase was added and the mixture was placed in a thermostat at 35°. An additional 2 mg. of the enzyme preparation was added after 4½ hours and again after 9 hours. Samples were taken for paper chromatography after 1, 4½ and 19 hours. The last sample was found to contain free arabinose. Prolonged treatment with pectase did not split off other monosaccharides.

Isolation of polysaccharides by direct precipitation with ethanol. Four hundred g. of the coarse capsule powder was extracted twice by maceration at 30 to 40°, first with 8 l. then with 4 l. of water for 2½ hours each time. The combined aqueous extracts were filtered and the filtrate concentrated to about 30 ml. under reduced pressure (25 mm. Hg). The polysaccharides were precipitated with ethanol and collected on a sintered glass filter.

The brown, granular substance was purified by dissolving it in water and precipitating with ethanol. This was repeated and the product dissolved in 1 per cent acetic acid and again precipitated with ethanol. As the precipitate was still dark brown in colour, it was dissolved in water and the solution macerated with charcoal at 30 to 40° for 2 hours. After filtration, precipitation with ethanol and drying, the polysaccharides appeared as a grayish powder. Yield: 5·3 g.

When dissolved in water in the proportion 1 : 20, the product gave a brown, turbid but not viscous solution with a pH of 6·0. The product did not reduce Fehling's solution, but gave positive test with Molisch's reagent. The naphthoresorcinol-hydrochloric acid test for uronic acids[10] was also positive. The equivalent weight, as determined by titration with alkali was near 1000. Hydrolysis with acid produced a mixture which showed the same qualitative composition with respect to carbohydrates as the hydrolysate described under water-soluble polysaccharides. However, no arabinose could be detected after treatment with pectase.

REFERENCES

1. Permanent Central Opium Board, United Nations, Geneva. Report to the Economic and Social Council on Statistics of Narcotics for 1953 and the Work of the Board in 1954. United Nations Publications, Sales Number, 1954, **XI**, 4.
2. Tollens-Elsner, *Kurzes Handbuch der Kohlenhydrate*, Leipzig, 1935, p. 87.
3. Tollens-Elsner, *ibid.*, p. 158.
4. Tollens-Elsner, *ibid.*, p. 112.
5. Tollens-Elsner, *ibid.*, p. 113.
6. Klevstrand and Nordal, *Acta chem. scand.*, 1950, **4**, 1320.
7. Bevenue and Williams, *Arch. Biochem. Biophys.*, 1951, **34**, 225.
8. Hough, Jones and Wadman, *J. chem. Soc.*, 1949, 2511.
9. MacIlvaine, *J. biol. Chem.*, 1921, **49**, 183.
10. Patridge, *Nature, Lond.*, 1949, **164**, 443.

COMPARATIVE EFFICACY OF BACTERICIDAL COMPOUNDS IN BUFFER SOLUTIONS

PART II

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A number of phenols, aromatic alcohols, organic mercury compounds and quaternary ammonium compounds have been examined, by the membrane filter method, to assess their antibacterial action at four different pH values against various Gram-positive and Gram-negative organisms. The killing rate of the compounds tested varied with the pH. The phenols, in the undissociated state, and the aromatic alcohols were most effective against Gram-negative cells at a slightly alkaline pH, and least effective at a pH of 5·5 to 7, whereas against Gram-positive cells, the compounds were least bactericidal at a pH of 7 to 8·5. Upon Gram-positive as well as Gram-negative cells, phenylmercuric borate was more potent in alkaline solution, thiomersal, in acid solution. This difference is connected with the opposite charges of the active ions. A reduction in bactericidal activity was noted in acid solutions of domiphen bromide and cetyl pyridinium chloride although the activity of the latter was less influenced by pH. The importance of the partition ratio between water and a lipid phase for the bactericidal efficacy of phenols and aromatic alcohols is stressed, and a new approach to the testing of these compounds is proposed. 4-Chloro-β-phenylethyl alcohol yielded promising results as a new bactericidal agent. *Pseudomonas pyocyanea* was more rapidly killed by the compounds tested than *Escherichia coli*.

IN Part 1 of this work[1], a technique for the counting of bacteria by means of membrane filters was described and analysed statistically. This technique has been used in a large number of experiments, the results of which are discussed in the following pages.

EXPERIMENTAL METHODS

The details of the technique employed as well as the compounds and organisms tested have been described in Part I[1]. The cell density was 3×10^5/ml.; 10 ml. of the test-mixture was filtered through Co 5 membrane filters which were incubated on enriched nutrient agar with 0·05 per cent of thioglycollate for mercurials; three filter pads were used for each pH. Figures based on a logarithmic scale are used to present the counts, each column representing the "mean" of the three replicates, and the highest as well as the lowest count is indicated.

RESULTS AND DISCUSSION†

Bactericidal Action of Phenols and Aromatic Alcohols

Influence of the pH. Figures 1 and 2 give the results obtained with *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas pyocyanea*, *Streptococcus faecalis*, *Serratia marcescens* and *Corynebacterium diphtheriae*

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† Only a small number of the counts obtained can be reproduced here. Values of n (concentration exponents) and Q_{10} (temperature coefficients), calculated on the basis of mortalities exceeding 99·9 per cent, may be summarised as follows: Phenols and aromatic alcohols: $n = 6$, $Q_{10} = 4{\cdot}3$ (calculated on the basis of the difference between 20° and 37°); mercurials: $n = 2$; quaternaries: $n < 2$.

tested against phenol and β-phenylethyl alcohol. For the Gram-negative species, the minimal action (that with most survivors) was found to be on the acid side, at about pH 5·5 for *E. coli*, and at pH 5·5 to 7 for *Ps. pyocyanea* and *Serr. marcescens*. For the Gram-positive cells, on the other hand, minimal action was at the slightly alkaline pH values of 7 to 8·5 whereas between pH 7 and 5·5, a large increase in bactericidal action was recorded. Similar results were obtained with the other phenols and aromatic alcohols tested; dissociating phenols, however, were found to behave differently.

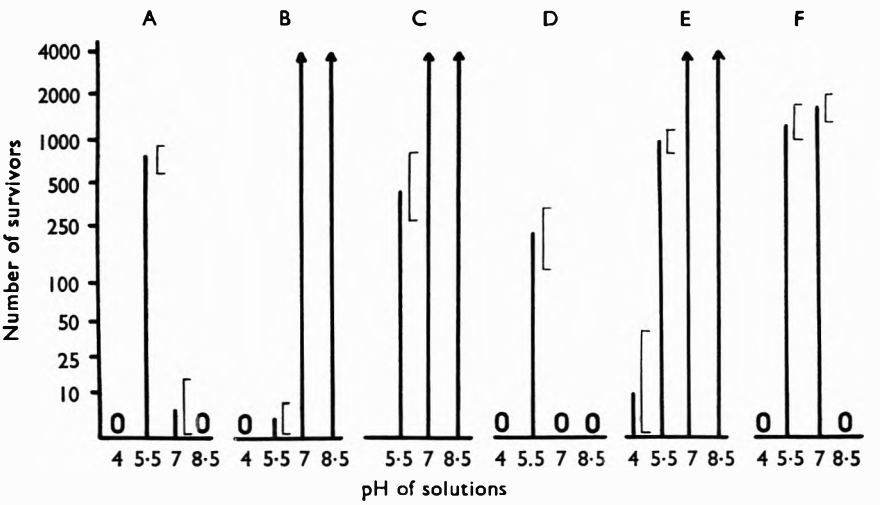


FIG. 1. Membrane filter counts of suspensions of *E. coli* (A, D), *Staph. aureus* (B, E), *Str. faecalis* (C) and *Ps. pyocyanea* (F), exposed to 1 per cent phenol (A, B, C) and 1 per cent (D, E) or 0·5 per cent (F) β-phenylethyl alcohol in isotonic phosphate buffers of four pH values. Each column represents the "mean" calculated on the basis of the logarithms of three parallel counts, brackets indicate lowest and highest counts. An arrow at the top of a column indicates filters too densely grown for a count of single colonies, the limiting count being 2000 to 3000 colonies for the Gram-negative organisms and about 4000 for the Gram-positive ones. For each count, 10 ml. of test-mixture with an initial count of $3 \cdot 10^5$ cells per ml. were filtered, 0 = sterile.

The results with the Gram-negative organisms are contrary to the axiom that phenols are more bactericidal in acid solutions. Especially surprising was that, in general, the efficacy was even more pronounced at pH 8·5 than at pH 4, despite all the phenols tested showing some dissociation at pH 8·5.

One explanation why this characteristic difference between Gram-positive and Gram-negative organisms has not been detected sooner might be that the methods hitherto used e.g., those of Kuroda[2], were not sufficiently sensitive; thus with the more sensitive membrane technique now available these differences are now seen.

As the efficacy of phenols and alcohols was influenced so characteristically by the pH of the solution, we first sought a clue in the different

isoelectric points of Gram-positive and Gram-negative bacteria discussed by Stearn and Stearn[3]. They measured the different uptakes of dye by the cytoplasmic membrane, but to us a difference in the charges of cytoplasmic membranes is difficult to reconcile with the fact that phenols are bactericidal in the molecular form only. It seems that no direct binding of the molecules to acceptor sites can be held responsible; rather, the permeability barrier may be influenced by the external pH. Thus, owing to the difference in barrier between Gram-positive and Gram-negative cells, the penetration of the disinfectants into these cells may not be the same at pH 5·5. Further studies in this field will be necessary to clarify this point.

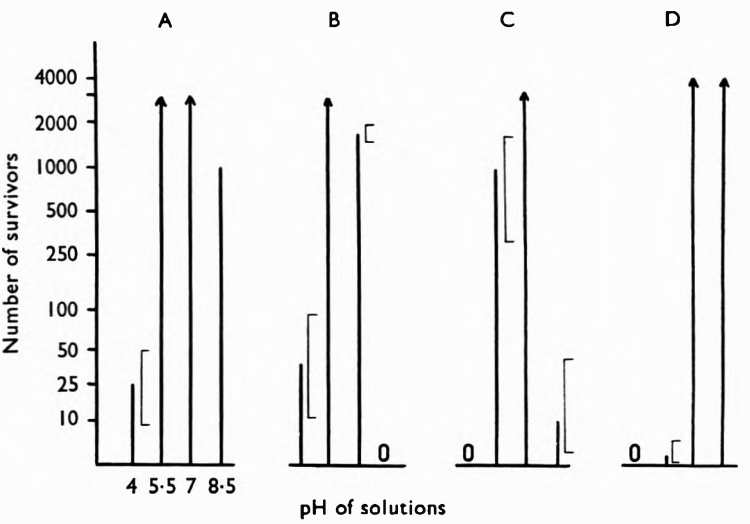


FIG. 2. Membrane filter counts of suspensions of *Serr. marcescens* (A), *Ps. pyocyanea* (B, C, two parallel series), and *Corynebact. diphtheriae* (D), exposed to 0·5 per cent of phenol for 2 hours at 20°. Phosphate buffers, cell density and representation as in Fig. 1.

Influence of the dissociation of phenols. It is well known that th dissociation of phenols increases with increasing pH value, and that this affects their antibacterial properties. This is illustrated in Figure 3 which indicates the bactericidal activities of *p*- and *o*-chlorophenol, propyl-*p*-hydroxybenzoate and *p*-nitrophenol at different pH values against *E. coli*. It shows the rise in dissociation to be proportional to the fall in bactericidal activity. Phenolate ions are therefore not bactericidal, which is in agreement with the statements of other authors. From the Figure, it is possible to estimate the quantitative aspect of the survivor peak at pH 5·5. *o*-Chlorophenol and propyl-*p*-hydroxybenzoate are 3 per cent dissociated at pH 7, which is sufficient to obtain about as many survivors as at pH 5·5. Small differences in concentration seem therefore to be sufficient for a shift in the survivor peak from 5·5 to 7 (or vice versa) with *E. coli*. *p*-Chlorophenol, on the other hand, is

almost 20 per cent dissociated at pH 8·5, with about 20 times as many survivors as at pH 5·5.

The part played by the partition coefficient in the arrangement of the compounds according to their activity. In a preliminary report[4] it was pointed out that our results with phenols and aromatic alcohols are in general agreement with Ferguson's principle[5,6]. This rule, however, stipulating that activity is inversely related to water solubility was not followed by the two chlorinated alcohols, namely, 4-chlorobenzyl alcohol and 4-chloro-β-phenylethyl alcohol. For instance, a 0·021M aqueous solution of 4-chlorobenzyl alcohol (water solubility = 0·021M) exhibited

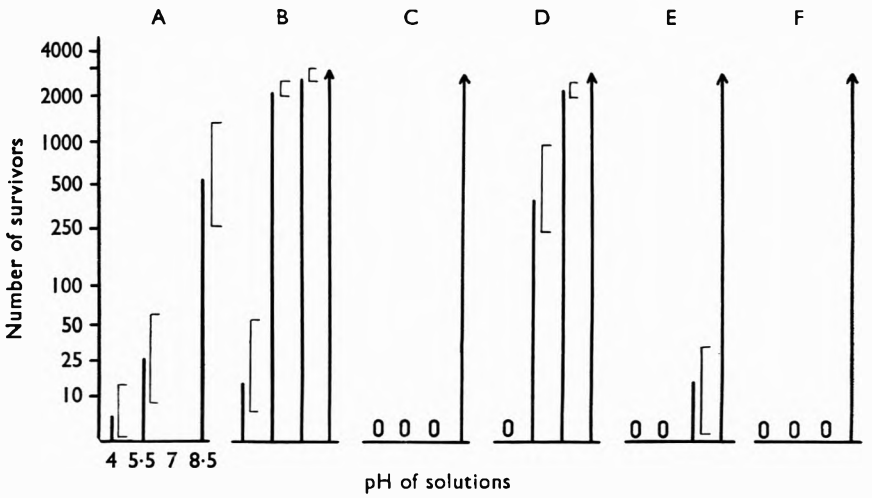


FIG. 3. Membrane filter counts of suspensions of *E. coli* exposed to phenols of varying degrees of dissociation; dissociation rises from left to right. A, *p*-chlorophenol 0·1 per cent for 6 hours at 20°: B, *o*-chlorophenol 0·25 per cent for 30 minutes at 20°, and for 2 hours at 20° in C; D, propyl *p*-hydroxybenzoate 0·03 per cent for 24 hours at 20°; E, *p*-nitrophenol 1 per cent for 30 minutes at 20°; F, *p*-nitrophenol 0·5 per cent for 24 hours at 20°. For buffers, cell density and representation: cf. Fig. 1.

the same bactericidal activity as 0·013M solution of 4-chloro-β-phenylethyl alcohol (water solubility = 0·0294M). However, when these solutions of equal activity were partitioned in hexane:water, 4-chlorobenzyl alcohol achieved a concentration of 0·0106M and 4-chloro-β-phenylethyl alcohol a concentration of 0·0091M in the hexane, the concentrations in the lipid phase thus being similar. Richardson and Reid[7] obtained similar results with α-ω-di-*p*-hydroxyphenylalkanes, as did Shukis and Tallman[8] with aliphatic mercurials. Crisp and Barr[9] confirmed Ferguson's rule giving data on the activities of alcohols and phenols required to immobilise barnacle nauplius larvae. They too stressed that the efficacy of phenols and alcohols is governed by the concentration reached in the biophase. This phase was felt to be a bulk lipid rather polar in character and not an interface between lipid and water.

Our experiments have convinced us that it is possible to use a simple approach to test phenolic and alcoholic disinfectants. Starting from the hypothesis that different concentrations of phenols and aromatic alcohols elicit an identical bactericidal activity when they reach the same molar concentration in a lipid phase after partitioning, reference to physical constants of the compounds in question will allow a comparison of the activities of different compounds to be made. The ratio molar concentration in water: molar concentration in lipid phase after partitioning, provisionally termed "activity index", is a constant for a given compound, provided the same lipid phase is used and that the phenols are undissociated. Once the "activity indices" have been determined experimentally, different compounds can be compared by calculating the concentrations in water which are required to obtain a similar concentration in the lipid phase. The calculated concentrations will probably not be strictly equal in their bactericidal activity since other factors are involved, nevertheless, this approach might be useful for a preliminary comparison.

It has long been known that upon ascending a homologous series, one observes, as a rule, the antibacterial activity to increase and then to decrease again. This fact, already explained in a simple manner by Ferguson[5], may be interpreted from our point of view as follows. On the one hand, the activity of the compounds augments with increasing solubility in lipids. On the other hand, the disinfectant has to be dissolved in water in order to reach the hydrophilic outer layers of the cells. In a homologous series, therefore a certain point will be reached where a further, significant increase in lipid solubility cannot be attained whilst at the same time the solubility in water is lowered, and this means that the activity is necessarily reduced. As Ferguson pointed out, the position of the fall in action depends on the resistance of the organisms; it occurs earlier with *Staph. aureus* than it does with *Salmonella typhi*, for example. One may predict that phenols and aromatic alcohols capable of reaching a high concentration in the cell lipids yet remaining soluble to some extent in water will possess superior killing power. Chlorocresol and 4-chloro-β-phenylethyl alcohol are examples of such compounds.

Our results are in favour of the existence of a lipid barrier at the cell surface. This barrier is very probably represented by the cytoplasmic membrane[10].

Bactericidal Action of Organic Mercury Compounds

Figure 4 shows the activities of phenylmercuric borate and thiomersal at four pH values. It is seen that whilst their activities differ greatly, their effects on the Gram-positive and Gram-negative organisms are identical. It is generally agreed that the mechanism of action of mercurials is an interaction with available sulphhydryl compounds since compounds such as thioglycollate can compete with the mercuric ion. The sulphhydryl group has a pKa of 9·1 to 10·8 at 25° in peptides of known structure[11]. According to Cohn and Edsall[11], the pI (isoelectric point) of glutathione is 2·83 and that of cysteine 5·02 to 5·07; because the more alkaline the

solution of these compounds, the greater their negative charges. The difference between the action of phenylmercuric borate (cationic) and thiomersal (anionic) is easily explained by the fact that in more alkaline solutions a greater number of positively charged phenylmercuric ions can be bound by the sulphhydryl groups; for the negatively charged thiomersal, the opposite is true. The interaction must, of course, occur at a site of the cell still influenced by the external pH. The action of mercurials is further elucidated by results like those of Shukis and Tallman[8], who showed that in a series of aliphatic mercurials, the antibacterial action was better when the compounds had a partition coefficient in favour of

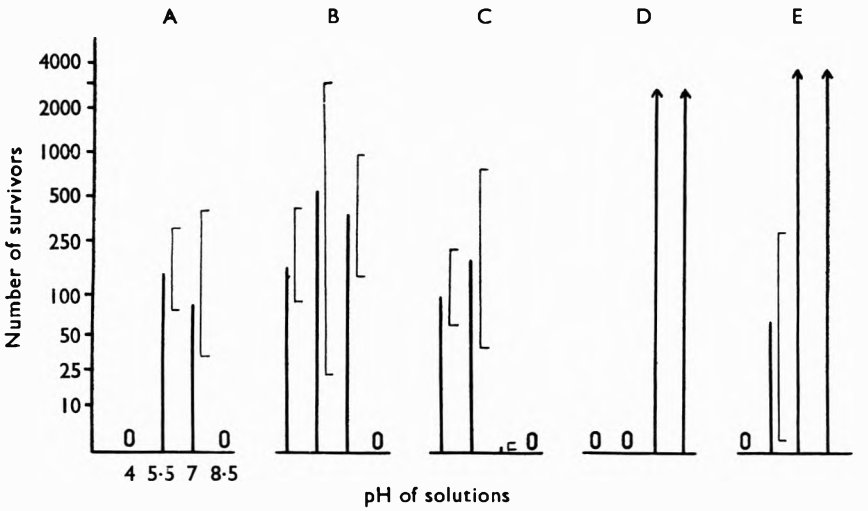


FIG. 4. Membrane filter counts of suspensions of *E. coli* (A, D), *Ps. pyocyanea* (C), and *Staph. aureus* (B, E), exposed to phenylmercuric borate 1:10,000 (A, B, C) and thiomersal 1:10,000 (D, E). Exposure times: 30 minutes at 20° in C, 2 hours at 20° in A and B, 24 hours at 20° in D and E. The phenylmercuric borate solutions were in M/15 phosphate buffers only, the thiomersal solutions were made with the usual isotonic phosphate solutions with potassium chloride. For cell density and representation: cf. Fig. 1.

the lipid phase. We found phenylmercuric borate with its higher solubility in lipids to be much more active than thiomersal which is poorly lipid-soluble.

The action of mercurials seems to proceed in two stages. In the first, the compound is adsorbed on to the cytoplasmic membrane, the adsorption being successful only when the charges of the interacting groups are opposite. The sulphhydryl groups attacked seem to be the same both in Gram-positive and Gram-negative species. The first stage is essentially bacteriostatic and is reversible by thioglycollate. In the second stage, penetration through the lipid barrier, situated most probably in the cytoplasmic membrane, takes place. For this penetration, the water:lipid partition coefficient of the compound is the deciding factor. Once the antiseptic has penetrated, the action is no longer reversible and is therefore bactericidal.

Bactericidal Action of Quaternary Ammonium Compounds

A comparison of the two compounds tested (Figs. 5 and 6) showed that cetyl pyridinium chloride was more active than domiphen bromide against *Staph. aureus*, 1 : 100,000 of the former compound being more effective in 2 hours than the same concentration of domiphen bromide in 24 hours. This difference was never noted with *E. coli*. While a more pronounced

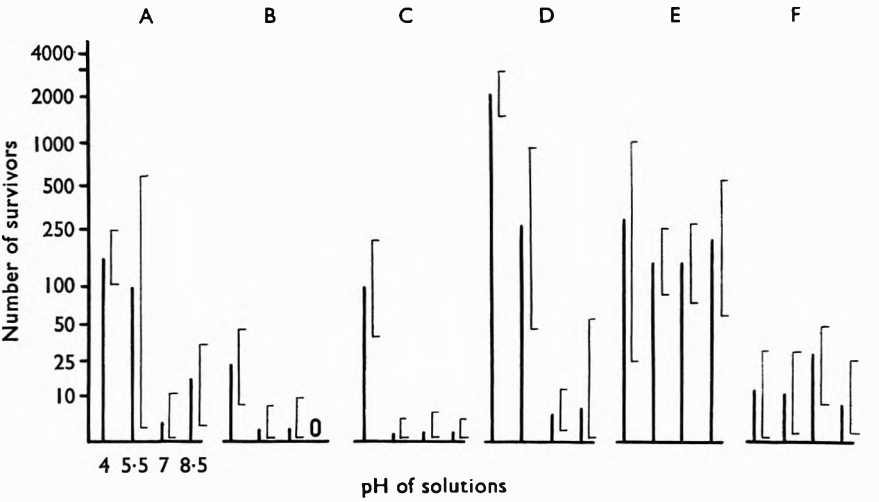


FIG. 5. Membrane filter counts of suspensions of *E. coli* (A, C, E) and *Staph aureus* (B, D, F) exposed to domiphen bromide 1 : 20,000 (A, B), 1 : 50,000 (C, D), and 1 : 100,000 (E, F), Exposure times: 30 minutes at 20° (B), 2 hours at 20° (A, C, D), and 24 hours at 20° (E, F). Experiments B and C were conducted with suspensions of *E. coli* of lesser resistance than those used in A. In experiments B and D, four parallel filtrations were made. For buffers, cell density and representation: cf. Fig. 1.

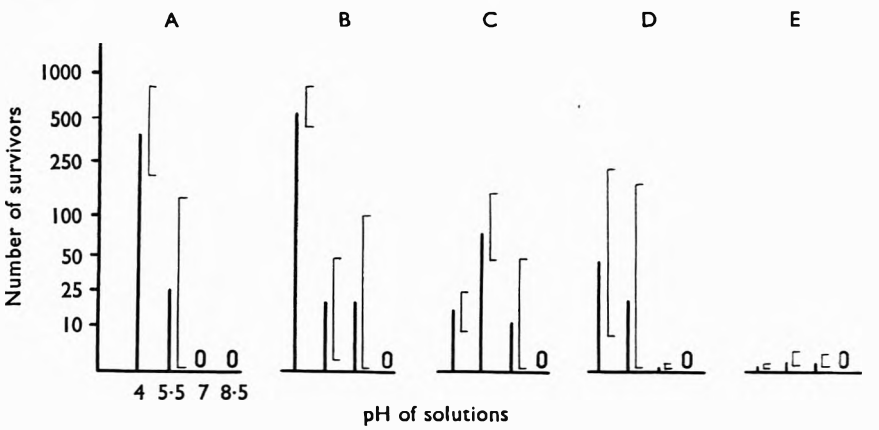
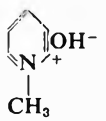
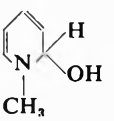


FIG. 6. Membrane filter counts of suspensions of *E. coli* (A, B) and *Staph. aureus* (C, D, E) exposed to cetyl pyridinium chloride 1 : 10,000 for 30 minutes at 20° in A, 1 : 20,000 for 2 hours at 20° in B, 1 : 100,000 for 2 hours at 20° in C, D and E (three parallel series). Buffers, cell density and representation same as in Fig. 1.

bactericidal action of the quaternaries in alkaline solutions is to be expected because of the positive charge of the active molecules, cetyl pyridinium chloride was reported by Quisno and Foter[12] to be equally active between pH 2 to 10. This is partly confirmed by our results (Fig. 6). In spite of its being a weak base (pKb 8·8), pyridine forms quaternary salts which act as strong electrolytes. The quaternary hydroxides, however, exhibit properties[13] that might possibly be able to furnish some explanation for the comparatively milder bactericidal activity at alkaline pH values. Thus, *N*-methylpyridinium hydroxide "a" responds to oxidation with potassium ferricyanide as though it had structure "b".



a



b (Pseudo base)

In alkaline solutions, more of the pseudo base is likely to be formed; this non-ionic compound probably being less bactericidal.

Most workers testing quaternaries have obtained erratic results (see Davies[14]). The explanations generally offered are irregular distribution or agglutination of the cells in the test medium. Combined agglutination and killing have also been advanced to explain the very rapid killing rate in the first few minutes[15,16], followed by a slowing down of the killing process. Davies[14] as well as Du Bois[17] ascribed the persistence of a few survivors to the possibility that cells within agglutinates could no longer be reached by the disinfectant.

To study this further we examined cultures of *E. coli* and *Staph. aureus* in solutions of pH 4 and 7 by phase contrast to see whether agglutination of cells occurred under our experimental conditions. The cell density had to be raised to 15×10^6/ml. for these examinations. *E. coli* was agglutinated by both compounds at 1 : 5000, but not at 1 : 10,000, whereas *Staph. aureus* was agglutinated by dilutions as high as 1 : 200,000. Although agglutination was demonstrated by this method, no differences in survivor counts were found when the cultures were shaken with beads in a lecithin-Tween solution. Our experiments showed the quaternaries to have a very potent bactericidal action which is probably due to the low density of the washed cells employed and to the absence of foreign matter which might act as surface-active adsorbent. The killing of most cells in the first few minutes of exposure is no doubt ascribable to the surface-activity of the quaternaries which are absorbed rapidly by the cytoplasmic membrane, causing disorder and changes in surface charges in this essential part of the cell[18,19]. An important factor seems to be a profound change in permeability. Chaplin[20] described strains of *Serr. marcescens* which were resistant to quaternaries, and he observed that these strains contained more lipids in the cell surface. Similar results were obtained by Dyar[21] with *Staph. aureus*. The persistence of a few survivors might be attributed to differences in the lipid content of the cell surfaces.

Comparison of the Resistance of the Organisms Tested

Our results confirm that *Staph. aureus* is generally more resistant to phenols and aromatic alcohols than *E. coli*. The few experiments with *Str. faecalis* indicate that it is even more resistant than *Staph. aureus*. *Ps. pyocyanea* was more susceptible than *E. coli* to both the phenols and aromatic alcohols as well as to the mercurials and quaternaries tested. However, *Ps. pyocyanea* is still generally regarded as an organism showing marked resistance to disinfectants. Our findings on the efficacy of the quaternaries agree with those of Lawrence[22] and with Ostrolenk and Brewer[23]. Klein and others[24] found the quaternaries to be less active although they used a different method. Berry[25], who tested various organisms against phenoxetol only, suggested that this compound is particularly effective against *Ps. pyocyanea*. However, results of our comparative experiments in which we tested benzyl alcohol, β-phenylethyl alcohol and phenoxetol against *Ps. pyocyanea* show that β-phenylethyl alcohol is the most effective compound. Hence we could not confirm Berry's claim and suggest that the good effect of phenoxetol may be because *Ps. pyocyanea* offers minimal resistance to phenols and aromatic alcohols. After testing a number of typical, freshly isolated strains of the main test organisms we remain convinced that a general high resistance of *Ps. pyocyanea* to disinfectants does not exist although the occurrence of certain highly resistant strains cannot be excluded.

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References

1. Hess and Speiser, *J. Pharm. Pharmacol.*, 1959, **11**, 650.
2. Kuroda, *Biochem. Z.*, 1926, **169**, 281.
3. Stearn and Stearn, *Univ. Missouri Studies*, 1927, **3**, No. 2, 1–84.
4. Hess and Speiser, *Experientia*, 1958, **14**, 362.
5. Ferguson, *Proc. Roy. Soc., Lond.*, 1939, B **127**, 387.
6. Allawala and Riegelman, *J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 93.
7. Richardson and Reid, *J. Amer. chem. Soc.*, 1940, **62**, 413.
8. Shukis and Tallman, *ibid.*, 1943, **65**, 2365.
9. Crisp and Barr, *International Congress of Surface Activity, Proc. Second Congr.*, London, 1957, **4**, 310.
10. Mitchell and Moyle, *J. gen. Microbiol.*, 1951, **5**, 981.
11. Cohn and Edsall, *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, 2nd Edn. Reinhold, New York, 1943.
12. Quisno and Foter, *J. Bact.*, 1946, **52**, 111.
13. Fieser and Fieser, *Organic Chemistry*, 3rd Edn. Reinhold, New York, 1956.
14. Davies, *J. Hyg., Camb.*, 1949, **47**, 271.
15. McCulloch, Hauge and Migaki, *J. Amer. vet. Med. Ass.*, 1948, **112**, 283.
16. McCulloch, Hauge and Migaki, *Amer. J. Publ. Health*, 1948, **38**, 493.
17. DuBois, *Soap and San. Chem.*, 1947, **23**, 139.
18. Hotchkiss, *Ann. N.Y. Acad. Sci.*, 1946, **46**, 479.
19. Gale and Taylor, *J. gen. Microbiol.*, 1947, **1**, 77.
20. Chaplin, *J. Bact.*, 1952, **63**, 453.
21. Dyar, *ibid.*, 1947, **54**, 17.
22. Lawrence, *J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 457.
23. Ostrolenk and Brewer, *ibid.*, 1949, **38**, 95.
24. Klein, Millwood and Walther, *J. Pharm. Pharmacol.*, 1954, **6**, 725.
25. Berry, *Lancet*, 1944, **2**, 175.

LETTER TO THE EDITOR

The Reaction of Bases with Chloroform

SIR,—Klemperer and Warren[1] observed a reaction between certain alkaloids, including strychnine and brucine, with chloroform and concluded that the latter had reacted with the bases to form the corresponding quaternary dichloromethochlorides. Caws and Foster[2] found that the bases actually reacted with impurities in the chloroform and showed that small amounts of methylene chloride and bromochloromethane present in B.P. chloroform were responsible. Williams[3] has recently shown that other bases including pyrrolidine and piperidine also react with the methylene halides in chloroform and he gave a stereochemical explanation of the preferential reaction with the dihalogen compounds. There is still some doubt, however, whether chloroform free from methylene halides would react with these bases.

It appears rather unlikely that a reaction between strychnine and chloroform in which a white precipitate is visible after refluxing for an hour or two would have escaped notice until quite recently and it is possible that the reacting methylene compounds were not present in chloroform as manufactured years ago. A sample of chloroform produced some years ago proved difficult to find but eventually a number of ampoules were obtained with dates of manufacture between 1927 and 1941. These had been stored away from light and the only purification carried out was a simple distillation to remove a dyestuff which was present. This would not be sufficient to fractionate any impurities such as methylene halides. Two of these samples (dated 1936 and 1941) were refluxed with strychnine for nine hours and no reaction occurred, the solution remaining clear and bright and the strychnine recovered after removal of the solvent gave the same figure for titratable base in aqueous solution as did the untreated material. These results were confirmed by Dr. G. E. Foster in a personal communication.

Two samples of chloroform (dated 1936 and 1939) were examined by gas chromatography after a preliminary fractionation and there was no evidence of the presence of methylene chloride or of bromochloromethane. Samples of recently manufactured B.P. chloroform had been found to contain both these impurities.

A sample of chloroform prepared in the laboratory by the action of bleaching powder on acetone did not react with strychnine after refluxing for nine hours.

TABLE I

REACTION BETWEEN STRYCHNINE AND CHLOROFORM

0·3 g. strychnine refluxed with 35 ml. chloroform for 9 hours

Origin of chloroform	Appearance of solution after reflux	Free base after reaction per cent	Base reacted per cent
Manufactured A 1936	clear	98·5	nil
Manufactured A 1941	clear	98·0	nil
Manufactured A 1958	cloudy with precpt.	87·3 (6 hours reflux)	11·2
Manufactured B 1958	cloudy with precpt.	82·5	16·0
Prepared in laboratory bleaching powder method	clear	98·5	nil
Prepared in laboratory bleaching powder method with potassium bromide present	clear	99·0	nil
	Assay of untreated strychnine	98·5	

A further sample prepared from bleaching powder to which had been added 1 per cent of potassium bromide also failed to react with strychnine. These results are shown in Table I.

When strychnine was refluxed with B.P. chloroform of recent origin which had been treated at the boiling point with bromine in a silica flask while irradiated with ultra-violet light for four hours 30 per cent of the strychnine reacted while only 17 per cent reacted with untreated chloroform under the same conditions. This can be explained by the conversion of methylene chloride to the more reactive bromo-compound[4].

It is concluded that strychnine does not react with chloroform made by the bleaching powder process even when bromine is present in appreciable quantities in the latter. Chloroform made by chlorination of methane or by the reduction of carbon tetrachloride would be expected to contain methylene chloride and if bromine is present possibly the bromochloride also. Since chloroform bleaching powder does not appear to contain these impurities it is likely to be the best material for use in the analysis of organic bases.

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References

1. Klemperer and Warren, *Chem. Ind.*, 1955, 1553.
2. Caws and Foster, *J. Pharm. Pharmacol.*, 1956, **8**, 790; 1957, **9**, 824.
3. Williams, *ibid.*, 1959, **11**, 400.
4. Huntress, *Organic Chlorine Compounds*, 1948, John Wiley, p. 526.