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THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

JOURNAL OF PHARMACY AND PHARMACOLOGY

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J. Pharm. Pharmacol., 1964, **16**, 641–646 Received April 13, 1964

Research Papers

Physical and biological changes in an artificial fat emulsion during storage

J. BOBERG AND I. HÅKANSSON

A soybean oil emulsion for parenteral nutrition has been stored for about 2 years at 4, 20 and 40°. During storage, the pH fell and the FFA concentration increased. The Michaelis-Menten constant (K_m) and the maximum velocity of the lipoprotein-lipase reaction (V_{max}) also increased. Gross particles were formed in the fat emulsion, and its toxicity increased with time. All these changes were most pronounced during storage at 40°.

A SOYBEAN oil emulsion for parenteral nutrition (Schuberth & Wretlind, 1961) has been investigated in dogs and rabbits by several authors. The freshly prepared emulsion has a low toxicity in dogs at doses up to 9 g/kg weight for periods of 4 weeks (Edgren, Hallberg, Håkansson, Meng & Wretlind, 1964). Furthermore, the elimination of this emulsion from the blood stream in dogs is similar to that of dog chylomicrons (Carlson & Hallberg, 1963). The emulsion has also proved to be a suitable substrate in the lipoprotein-lipase reaction (Boberg & Carlson, 1964).

During the past 3 years this emulsion has been used in clinical practice (Schuberth & Wretlind, 1963) but it has not been subjected to systematic studies of the physico-chemical and biological changes occurring as a result of lengthy storage under various conditions. We now describe some physico-chemical changes observed during long-term storage at different temperatures, and also the physiological effect of these changes.

Experimental

Emulsion. The emulsion* contains 10% soybean oil, 1·2% egg phosphatides and 2·5% glycerol in the aqueous phase. One batch was transferred to bottles containing 100 ml. The bottles were sterilised by autoclaving, and stored in the dark at 4, 20 and 40°. The longest storage period was more than 2 years. After various intervals, the required number of bottles, were withdrawn and tested.

pH determination. Measurements were made with a glass electrode.

Determination of free fatty acids. Free fatty acids (FFA) were assayed in duplicate by the method of Dole (1956), as modified by Traut, Estes & Friedberg (1960).

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* Intralipid, kindly supplied by Vitrum AB, Stockholm.

Estimation of lipoprotein-lipase activity. FFA released during incubation of enzyme plasma and substrate *in vitro* was titrated according to Boberg & Carlson (1964). Plasma containing lipoprotein lipase was obtained by withdrawing blood from a fasting, healthy human donor, 1 hr after intravenous injection of 50 mg of heparin. The blood was centrifuged at 1000 *g* for 15 min. Plasma was drawn off, and frozen in tubes at −20°. The emulsion was used as substrate in the lipoprotein-lipase reaction. The lipoprotein-lipase activity was determined at three different substrate concentrations, and plotted according to Lineweaver & Burke (1934).

Particle-size determination. The particle size was measured under a microscope. The emulsion was diluted 1:25 with a 50% solution of propylene glycol in water, as described by Levius & Drommond (1953). The number of particles with a diameter of more than 0·5 μ was counted within one square of a ruled eyepiece scale. Ten squares were examined in each slide.

Animal experiments. Albino rabbits of either sex, weighing about 2·5 kg, were used. In each experiment, 20 ml of the emulsion, warmed to body temperature, was infused into the marginal ear vein over 25 sec. Six rabbits were injected with each emulsion in every test, and observed for one day. In those that did not survive, death occurred within less than 5 min.

Results

pH changes. Fig. 1 shows that pH fell during storage. The fall was most pronounced during the first 3 months. There was a significant difference between the pH of the emulsion stored at 4° and that stored at 40°.

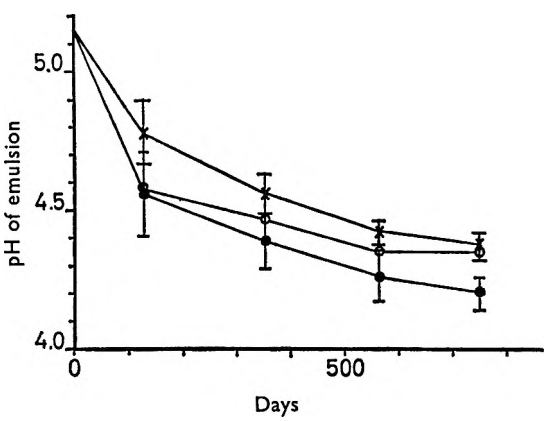

FIG. 1. Changes in pH during storage of emulsion at ×, 4°; ○, 20°; ●, 40° (mean ± S.E.).

Changes in FFA concentration. A spontaneous release of FFA occurred during storage which was practically linear with time (Fig. 2). Up to

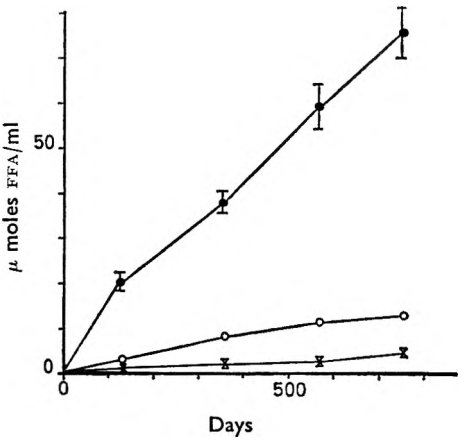

FIG. 2. Release of FFA during storage at ×, 4°; ○, 20°; ●, 40° (mean ± s.e.).

20° the FFA concentration was not high but in emulsions stored at 40° the release was much increased, for example, after 700 days storage the the FFA content of this emulsion was about 15 times greater than that of emulsions stored at 4°.

Properties of the emulsion as a substrate. Fig. 3 shows graphs of the lipoprotein-lipase reaction plotted according to Lineweaver & Burk (1934). The Michaelis-Menten constant (K_m) and the maximum velocity of the reaction (V_{max}) constant are listed in Table 1. In the emulsions stored at 4° and 20°, there were no significant changes with time. In those stored at 40°, both K_m and V_{max} increased significantly during the period studied.

TABLE 1. THE CONSTANTS K_m AND V_{max} CALCULATED ON THE BASIS OF THE CURVES IN FIG. 3

Test time, days	0		42		128		400	
Storage temp °C	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
4	0·84	0·178	0·52	0·206	0·37	0·140	1·00	0·135
20	0·87	0·180	0·16	0·144	0·74	0·080	2·54	0·285
40	0·91	0·192	0·44	0·148	5·56	0·572	20·03	1·012

K_m = Michaelis-Menten constant expressed in μ moles triglycerides/ml.
V_{max} = Maximum velocity of the reaction expressed in μ moles/min and ml.

Changes in particle size. At zero time the optical diameter of all particles was less than 0·5 μ, i.e. they could not be measured with the method used. During storage a few particles of 3 μ diameter were formed, but by far the greatest number counted were between 0·5–1 μ. The grossest particles were found in the emulsion stored at 40°.

Animal experiments. All the rabbits survived emulsion stored at 4° and 20°, whereas that stored at 40° showed an increasing lethal effect with time (Fig. 4).

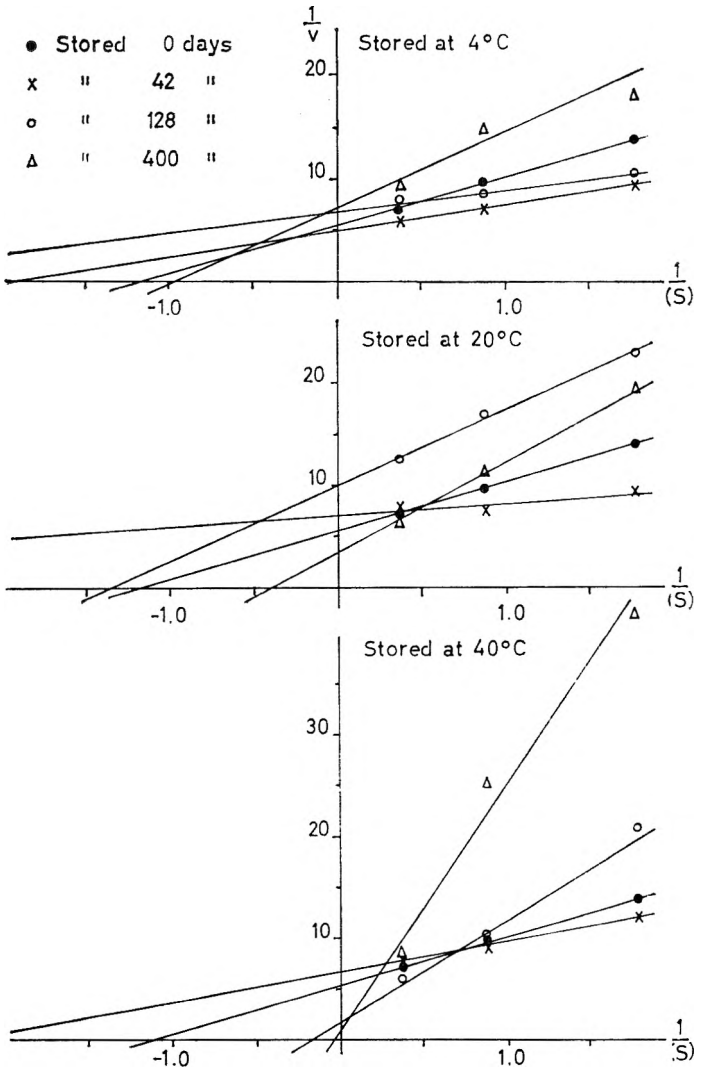

FIG. 3. Lipoprotein-lipase activity (LLA) tested on the emulsions plotted as straight lines according to Lineweaver & Burk. The equation of the straight line is:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{(S)}$$

where K_m is the Michaelis-Menten constant and V_{max} the maximum velocity of the reaction. y intercept $= \frac{1}{V_{max}}$ and x intercept $= -\frac{1}{K_m}$. The reaction rate (v) is μ moles FFA formed per min and ml post-heparin plasma. Substrate concentration (S) is μ moles triglycerides per ml test solution.

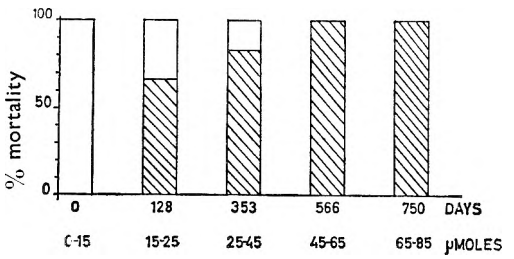

FIG. 4. Toxicity of the emulsion stored at 40° tested in the rabbit. Abscissa: Storage time in days and range of FFA concentration in μ moles/ml emulsion. Each column represents emulsion tested in 6 rabbits. Hatched area = mortality.

Discussion

It may be concluded that the pH falls with time, the FFA concentration increases with time, as do the constants K_m and V_{max}, and gross particles are formed. All these changes are more pronounced at 40°, when after 400 days, the pH falls from 5·2 to 4·4. The FFA concentration shows a five-fold increase from 0·8 to about 40 μ moles/ml. The K_m value is about 20 times higher than in the fresh emulsion, and the V_{max} value about 5 times higher. Moreover, this emulsion becomes toxic, and causes more than 80% mortality when injected into rabbits.

When the logarithm of the FFA concentration was plotted against the pH of the emulsion at each temperature, an almost linear correlation was present between these two variables. This suggests that as the fall in pH is almost linear to some extent it might be due to the increased concentration of FFA.

It is likely that the lipoprotein-lipase attacks the substrate on the surface of the particles. The increased K_m value in the lipoprotein-lipase reaction during storage may be due to the loss of affinity for the enzyme at this site. The formation of gross particles in the emulsion is probably of some importance in this respect, because, in relation to their fat mass, the surface of the gross particles is less. This necessitates the use of a higher substrate concentration to reach a zero order reaction of the lipoprotein-lipase reaction.

The only parameter of the emulsions studied, that can be correlated to the increased toxicity of the emulsion stored at 40° is the FFA concentration. This is in agreement with the findings in mice reported earlier by Orö & Wretlind (1961).

The cause of death in the rabbits is unknown but it may be of relevance in this context that injection of small amounts of saturated FFA causes massive thrombosis in dogs (Connor, Hoak & Warner, 1963).

Later unpublished investigations have shown that the liberation of FFA from soybean oil emulsion is minimum when the pH is between 6 and 7.

To conclude, our results indicate that the fat emulsion can be stored satisfactorily at a temperature of 4° for up to 2 years.

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J. Pharm. Pharmacol., 1964, **16**, 647–654 Received May 15, 1964

The effects of blocking agents upon the isolated vas deferens of the guinea-pig stimulated by the hypogastric nerve

A. B. MORRISON AND M. W. PARKES

When the guinea-pig isolated vas deferens preparation was stimulated by its hypogastric nerve, the responses obtained at different frequencies of stimulation were all reduced proportionately by guanethidine, bretylium, reserpine and pentamethonium. The difference between guanethidine and bretylium in affecting the responses to nervous stimulation at different frequencies of the cat nictitating membrane reported by Boura & Green was not observed with this preparation. Papaverine reduced responses to high frequencies of stimulation more than those to low frequencies. Amphetamine also reduced responses to all frequencies uniformly except in the smallest doses used, which potentiated the responses to high frequencies; these doses reversed the reduction in response by guanethidine, bretylium and reserpine. Preparations stimulated between parallel electrodes responded similarly to those stimulated by the hypogastric nerve.

THE isolated preparation of the guinea-pig vas deferens and hypogastric nerve as described by Hukovic (1961) responds to the adrenergic neurone blocking action of guanethidine and bretylium by a reduction in height of the contraction caused by electrical stimulation of the nerve (Boyd, Chang & Rand, 1961; Bentley, 1962). The technique of stimulating the nerve over a range of frequencies has been shown to distinguish between the blocking actions of guanethidine and bretylium when applied to the responses of the cat nictitating membrane to stimulation of the post-ganglionic sympathetic nerve of the superior cervical ganglion (Boura & Green, 1962). The study described here examined the results of applying this technique to the blocking action of these and other drugs on the vas deferens preparation.

Methods

The organ was prepared as described by Hukovic (1961) and set up in a 15 ml overflow-type gut bath. The preparation was bathed in Krebs solution (33 g NaCl; 17·5 ml 10% KCl; 14 ml 10% $CaCl_2$; 10·4 g glucose; 10·5 g $NaHCO_3$; 0·81 g KH_2PO_4; 1·47 g $MgSO_47H_2O$; in 5 litres of distilled water), which was bubbled with 95% O_2 and 5% CO_2 and maintained at 35°. Stimulation of the hypogastric nerve was effected by a shielded electrode around which the nerve was wound twice and the free end secured to the edge of the bath to prevent movement of the nerve on the electrode. The electrode was placed on the surface of the perfusion fluid. In some experiments the organ was stimulated between parallel electrodes completely immersed in the bath, following the method described as transmural by Birmingham & Wilson (1963). In these experiments, the vas deferens was cleaned of mesentery as closely as possible. With both methods of stimulation, the pulses were 2 msec wide and of

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supramaximal voltage. The nerve was stimulated for between 2 and 5 sec every minute, while stimulation between parallel electrodes was applied for 10 sec every 90 sec.

The frequency was varied within the range 4 to 32 pulses/sec; this usually elicited the complete range of response of the organ. It was found advantageous to have more observations at the lower than at the higher frequencies. If guanethidine, bretylium or reserpine were given in sufficient concentrations to produce a moderate reduction in the response, the response subsequently continued to fall progressively and did not level out. It was, therefore, necessary to make some allowance for this in the design of the experiment. In some cases it was possible to limit the fall by washing out the drug after a given interval, when a constant response was obtained.

In all these experiments, two series of frequency change were made for each standard and treatment, the frequency first being lowered progressively and then raised. This process was repeated after each addition of drug, time being allowed for equilibrium to occur. On changing the frequency, time was allowed for the response to settle to that determined by the new frequency. The frequency was not changed until at least three or four constant responses were obtained.

Results

The change in height of response of the preparation with change in frequency is shown in Table 1 and Fig. 1 *et seq.* The corresponding

TABLE 1. RESPONSE OF GUINEA-PIG ISOLATED VAS DEFERENS TO SUPRAMAXIMAL STIMULATION OF THE HYPOGASTRIC NERVE AT VARIOUS FREQUENCIES (Figures from typical experiment)

Frequency (shocks/sec)	Height of response (mm)*		
	On descending run	On ascending run	Mean
24	85	84	84·5
20	80	79	79·5
16	70	71	70·5
12	60	56	58
10	49	50	49·5
8	39	38	38·5
6	28	25	26·5
4	8	—	8

* Each figure quoted is the mean for the last three responses obtained at that frequency of stimulation.

change with frequency after addition of guanethidine to the bath, in amounts between 0·3 and 1·5 μg/ml is also shown in Fig. 1. The curve is typical of those obtained in seven such experiments and shows that a similar degree of depression is exerted over the whole frequency range. Similar relations between frequency and reduction of response were found for bretylium, in concentrations between 0·5 and 2·0 μg/ml (4 experiments; Fig. 2) and reserpine at 5 to 10 μg/ml (5 experiments; Fig. 3). The depression with reserpine was slow to develop and persistent; the

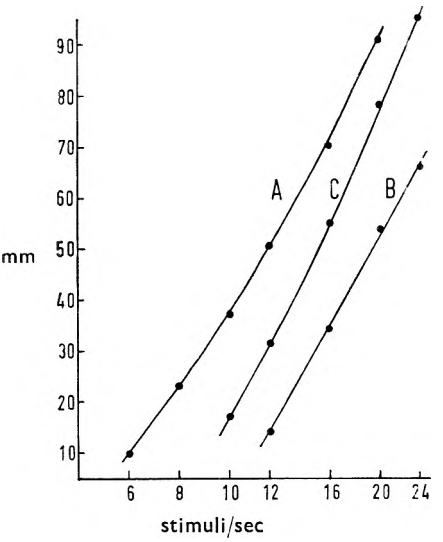

FIG. 1. Mean height of response, in mm of guinea-pig isolated vas deferens, stimulated by the hypogastric nerve with supramaximal shocks at various frequencies; from a single preparation. A. Before drug treatment. B. In the presence of guanethidine sulphate, 0·5 μg/ml. C. In the presence of amphetamine sulphate, 0·5 μg/ml following guanethidine sulphate, 0·5 μg/ml.

initial effect was to heighten the response, as also seen with lower, non-blocking doses (1 to 2 μg/ml). Papaverine however, affected the responses to high frequency stimulation more than those to lower frequency (Fig. 4).

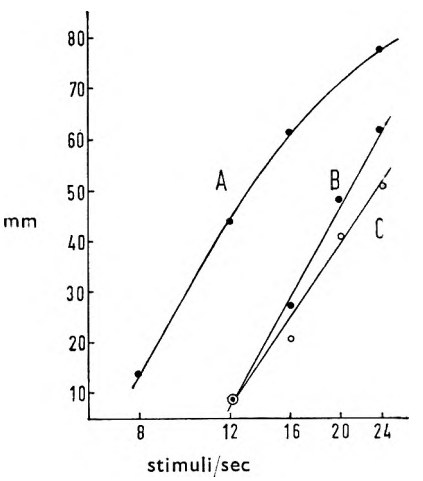

FIG. 2. Mean height of responses, in mm of guinea-pig isolated vas deferens, stimulated by the hypogastric nerve with supramaximal shocks at various frequencies; from a single preparation. A. Before drug treatment. B. In the presence of bretylium tosylate, 0·5 μg/ml. C. In the presence of pentamethonium iodide, 10 μg/ml.

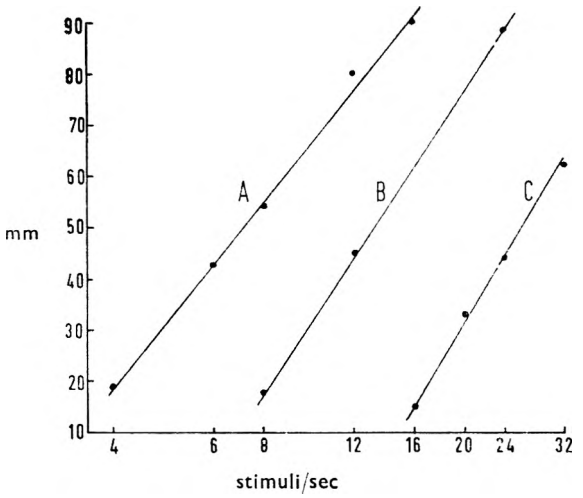

FIG. 3. Mean height of responses, in mm of guinea-pig isolated vas deferens, stimulated by the hypogastric nerve with supramaximal shocks at various frequencies; from a single preparation. A. Before drug treatment. B. 20 min after addition of reserpine, 5 μg/ml. C. 1 hr after washing out.

The ganglion-blocking agent, pentamethonium, at 10 μg/ml (Fig. 2) had a depressant action which was exerted similarly at all frequencies.

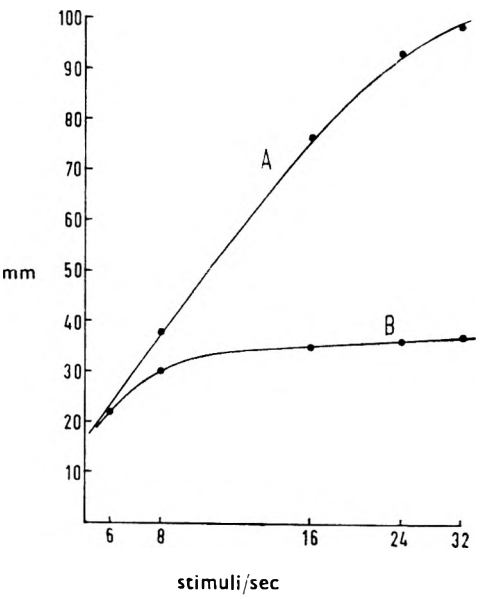

FIG. 4. Mean height of responses, in mm of guinea-pig isolated vas deferens, stimulated by the hypogastric nerve with supramaximal shocks at various frequencies; from a single preparation. A. Before drug treatment. B. In the presence of papaverine hydrochloride, 0·1 mg/ml.

In a few experiments in which the organ was stimulated between parallel electrodes alternately with stimulation of the hypogastric nerve, it was found that the reduction by hexamethonium of responses elicited by nerve stimulation, was greater than the reduction of those induced by stimulation between parallel electrodes, whilst both were equisensitive to guanethidine and bretylium (Fig. 5). Guanethidine and bretylium

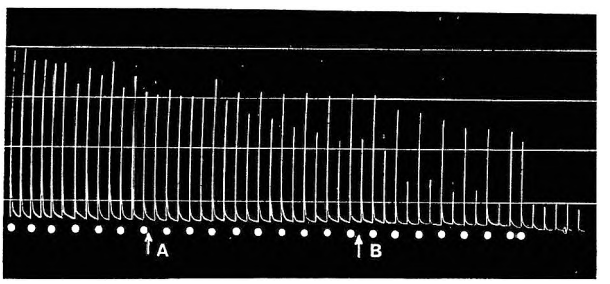

FIG. 5. Portion of kymograph record, guinea-pig isolated vas deferens, supramaximally stimulated by electrodes on the hypogastric nerve (unmarked) alternately with stimulation between parallel electrodes in the bath (marked). At A, addition of hexamethonium iodide to make a bath concentration of 5 μg/ml. At B, replacement with guanethidine sulphate to make a bath concentration of 0·6 μg/ml.

depressed the frequency-response curve in a parallel manner in preparations stimulated between electrodes as well as in the nerve-stimulated preparation, and similar doses of these drugs were required for reduction of the responses to both (Fig. 6; 4 experiments).

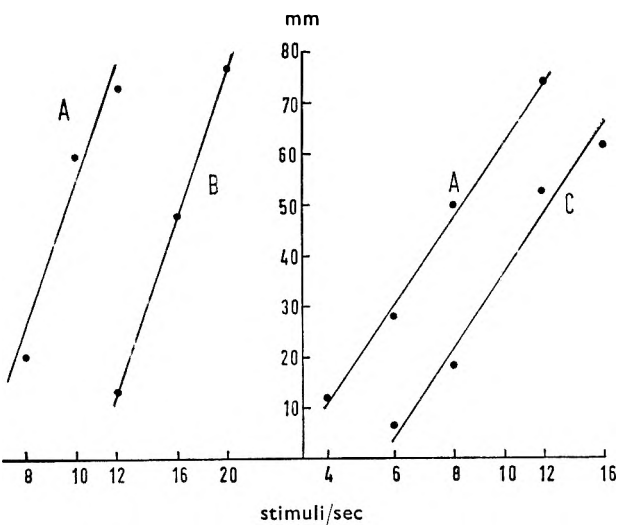

FIG. 6. Mean height of responses, in mm, of guinea-pig isolated vas deferens, stimulated between parallel electrodes with supramaximal shocks at various frequencies; each pair of curves from a single preparation. A. Before drug treatment. B. In the presence of guanethidine sulphate, 0·5 μg/ml. C. In the presence of bretylium tosylate, 1·5 μg/ml.

Amphetamine reduced the responses at concentrations above 10 μg/ml, but lower concentrations (0·5 to 5·0 μg/ml) potentiated the response to the higher frequencies of stimulation although reducing the response to lower frequencies (Fig. 7; 3 experiments). When added to the bath after

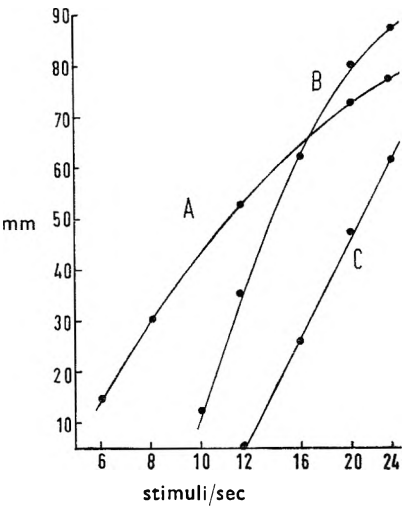

FIG. 7. Mean height of responses, in mm of guinea-pig isolated vas deferens, stimulated by the hypogastric nerve with supramaximal shocks at various frequencies; from a single preparation. A. Before drug treatment. B. In the presence of amphetamine sulphate, 0·5 μg/ml. C. In the presence of amphetamine sulphate, 20 μg/ml.

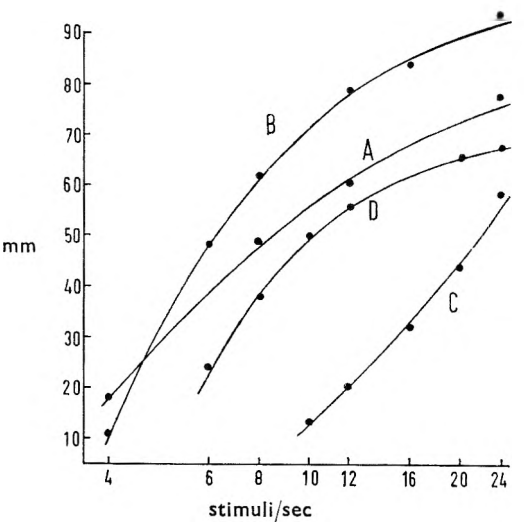

FIG. 8. Mean height of responses, in mm of guinea-pig isolated vas deferens, stimulated by the hypogastric nerve with supramaximal shocks at various frequencies; from a single preparation. A. Before drug treatment. B. In the presence of dibenamine hydrochloride, 10 μg/ml. C. In the presence of guanethidine sulphate, 0·33 μg/ml. D. In the presence of dibenamine hydrochloride, 20 μg/ml. following C.

depression of the response by guanethidine, amphetamine at 0·5 μg/ml restored the response at all frequencies (Fig. 1; 3 experiments). Amphetamine similarly reversed the block due to bretylium though the lower frequencies were less well restored than the higher ones. Amphetamine also reversed the block caused by reserpine; in this case, however, only the higher frequencies were restored, the lower ones being further reduced. This was very similar to the picture presented by amphetamine itself.

The adrenergic blocking agent, dibenamine, at 10 μg/ml, also potentiated the response to stimulation, predominantly at high frequencies (Fig. 8). Dibenamine also reversed the depression due to guanethidine (Fig. 8); the reversal was, however, of the same non-parallel form as the potentiation caused by dibenamine alone. It was noticed that there was a transient biphasic effect when dibenamine was added to the bath containing guanethidine, as there was initially a further depression before reversal of the guanethidine block.

Discussion

Investigation of effects upon the relation between response and frequency of stimulation may provide information on the mode of action of agents affecting the response. The approximately parallel degree of reduction over the range of frequencies found with guanethidine, bretylium and reserpine suggest uniform interference with the transmitter, either by competitive occupation of receptors or by reduction in the amount released. The gradual development and persistence of block due to reserpine, and the opposite action observed initially, recall the features of other actions of reserpine associated with amine depletion.

Boura & Green (1962) were able to distinguish between the action of bretylium and guanethidine in their effects on the cat nictitating membrane, stimulated by its sympathetic nerve, in that the frequency-response curve was depressed in a parallel manner by guanethidine, whereas bretylium had little effect on the response to low frequencies. From this they suggested that block due to guanethidine depended upon a competitive antagonism while the basis of the action of bretylium was non-competitive. In the results with the isolated vas deferens reported here, both guanethidine and bretylium appeared to depress the frequency-response curve in a parallel fashion, so that both would seem to exert a similarly competitive block.

A similar lack of differentiation between the blocking actions of guanethidine and bretylium has recently been found by Green & Robson (1964) using the isolated Finkleman preparation of the rabbit ileum, whereas the actions of these drugs on the stimulated responses of the vessels of the cat leg, the cat spleen and the vessels of the isolated rabbit ear resembled those on the nictitating membrane. A reason for the differences does not readily appear from consideration of the preparations concerned.

There is evidence that the sympathetic innervation of the vas deferens may not be entirely post-ganglionic (Sjöstrand, 1962a), supported by the

demonstration that ganglionic blocking agents reduce the response to stimulation of the hypogastric nerve (Sjöstrand, 1962b; Ohlin & Stromblad, 1963), a finding confirmed in this report.

Recent work has suggested that when the vas deferens is stimulated, either between parallel electrodes in the bath or transmurally (Birmingham & Wilson, 1963; Bentley & Sabine, 1963), this leads to stimulation of post-ganglionic fibres only, whereas hypogastric nerve stimulation involves preganglionic stimulation also. Results reported here confirm this evidence, in that while both means of stimulation were to some extent susceptible to ganglionic blockade, the responses due to hypogastric nerve stimulation were noticeably more reduced than those to stimulation between bath electrodes. Both, however, were equally sensitive to guanethidine. Bretylium and guanethidine affected the frequency-response curve in preparations stimulated between electrodes in the same manner as they did in those stimulated by the nerve, and thus the behaviour of the two drugs on the vas deferens does not seem to be related to the question of whether the stimulation is pre- or post-ganglionic.

The antagonism by amphetamine towards the block due to guanethidine reported by Day & Rand (1963) is confirmed in these experiments and its competitive basis is further suggested by frequency-response relations. Amphetamine given alone exerted a definite potentiating action in smaller doses. This might have contributed to reversal of the effects of guanethidine upon higher frequency responses but would not account for antagonism at lower frequencies, since in these circumstances amphetamine never potentiated responses.

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Some aspects of the mode of action of chlorhexidine

W. B. HUGO AND A. R. LONGWORTH

Chlorhexidine is rapidly adsorbed by bacterial cells and this adsorption is accompanied by other cytological changes which include changes in the permeability of the cells and in their optical properties. The amount of drug adsorption causing maximum leakage of cell constituents and changes in extinction was found to be equivalent for *Escherichia coli* and for *Staphylococcus aureus*. Higher doses of chlorhexidine causing a higher level of drug adsorption caused correspondingly less leakage and change in extinction although such higher doses were more rapidly bactericidal.

CHLORHEXIDINE B.P. is a potent antibacterial compound the properties of which were first described by Davies, Francis, Martin, Rose & Swain (1954).

Experiments determining drug uptake, leakage of cell constituents and turbidity changes designed to elucidate the mode of action of this compound are described herein.

Experimental

MATERIALS

Chlorhexidine diacetate (I.C.I. Ltd.) was used throughout. This salt has a solubility in water of 1·9% at 20°.

Culture media were prepared, except where stated, from Oxoid reagents. The nutrient broth contained %: Lab Lemco 1·0, peptone 1·0, sodium chloride 0·5, distilled water to 100 ml. Lubro-lecithin broth was prepared from this by the addition of %: Lubrol W (I.C.I.) 1·0, egg lecithin 95/100% (B.D.H.) 0·5. In addition, when using *Escherichia coli* the medium contained %: lactose 1·0 and for *Staphylococcus aureus*, glucose 1·0. Nutrient agar was prepared by addition of 1·8% Agar No. 3 to nutrient broth. The pH of all media after adjustment and sterilisation was 7·3. The organisms used were *E. coli* Type 1, formerly NCTC 5934, and *Staph. aureus* (Oxford strain).

METHODS

Suspensions of organisms were prepared by washing 18 hr growth from the surface of nutrient agar, the suspension, after centrifugation at low speed to remove agar fragments, was washed twice by recentrifuging (8,000 *g*, 10 min) before final suspension in distilled water or phosphate buffer (0·013 M, pH 7·3) and standardised nephelometrically against a previously constructed calibration curve.

Chlorhexidine was determined by the method of Holbrook (1958), or by measuring the absorption at 232 or 252 mμ. Drug uptake (adsorption) was determined by allowing cells to remain in contact with drug solutions under varying conditions of time and pH, centrifuging down the cells and determining residual chlorhexidine in the supernatant fluid after further clarification by centrifugation (8,000 *g*, 10 min).

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For short contact periods the method of Holbrook (1958) could be used to determine residual chlorhexidine, but in general, due to leakage of cellular material, the accuracy of Holbrook's method was seriously impaired. Consequently, chlorhexidine was extracted from the clarified supernatant, after addition of an equal volume of sodium hydroxide solution (0·1 N), by shaking with 4 aliquots of chloroform. The combined chloroform extracts were evaporated to dryness *in vacuo*, the residue taken up in acetic acid solution (0·1%), and chlorhexidine determined in this solution by measuring the absorption at 252 mμ. Cellular exudate absorbing at 260 mμ was determined on the aqueous phase from this extraction after first warming to remove dissolved chloroform.

Pentoses in the cellular exudate were determined by the method of Mejbaum (1939) on a separate aliquot of the supernatant.

Turbidity changes in the suspensions were determined by measuring changes in absorption at 500 mμ, at which wavelength extinction changes are due to differences in light scattering only (Mitchell, 1950). All spectrophotometric determinations were made on a Unicam SP500 spectrophotometer.

The bactericidal effect of chlorhexidine was determined by calculating the mean single survivor time (MSST) by the method of Mather (1949) from extinction data derived according to the method of Berry & Bean (1954) employing 20 replicates and a quenching volume of Lubrol-lecithin broth of 5 ml which was shown to neutralise residual chlorhexidine satisfactorily. Tubes were examined for growth by visual examination and as indicated by acid production detected by addition of phenol red after 48 hr incubation at 37°. This method of estimating bactericidal activity was chosen as, according to the authors, it is the most reliable one where agglutination tends to occur.

Results

The form of the isotherm for chlorhexidine uptake by *E. coli* and *Staph. aureus* suspended in distilled water and phosphate buffer (0·013 M, pH 7·3) after 10 min at 20° is shown in Fig. 1 and corresponds to the L type of Giles, MacEwan, Nakhwa & Smith (1960). This is indicative of a situation where the adsorbate molecules are at first readily taken up by the adsorbing species, but as the sites become filled the chance of further adsorption slowly decreases. Such a situation is found, for example, with most surface-active compounds on a variety of substrates. The steepness of the initial part of the curve suggested that the affinity of chlorhexidine for the sites on the cells of both species is high.

The effect of pH on the adsorption process is shown in Fig. 2, where under the conditions specified the amount of drug adsorbed increases with increasing pH. This curve might reflect the effect of changing pH both on the state of ionisation of the cell surface and of the drug itself. The dissociation constants (pK_a values) of chlorhexidine are 10·3 and 2·2 (Taylor, P. J., personal communication), corresponding to the formation of a mono- and di-cation. At pH 2, where little or no uptake

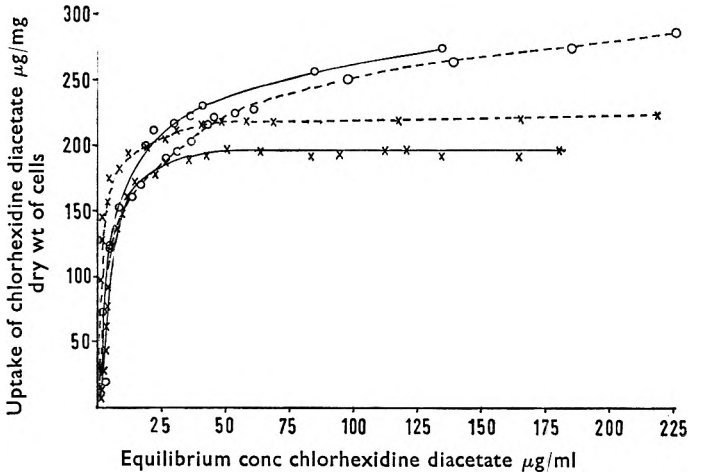

FIG. 1. Adsorption isotherms for the uptake of chlorhexidine diacetate by *E. coli* ——— and *Staph. aureus* – – – – suspensions 0·6 mg dry wt cells/ml from solutions of chlorhexidine diacetate after 10 min at 20°. —×—, Adsorption from aqueous solution. —○—, Adsorption from 0·013M phosphate buffer pH 7·3.

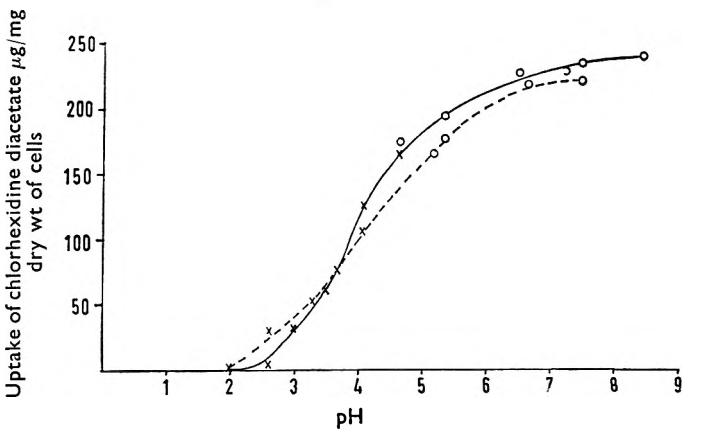

FIG. 2. Effect of pH on adsorption of chlorhexidine diacetate by *E. coli* (———) and *Staph. aureus* (– – – –) suspensions 0·6 mg dry wt cells/ml from M/28 buffer solutions containing 200 μg/ml chlorhexidine diacetate after 10 min contact at 20°. —×—, McIlvaines citrate-phosphate buffer. —○—, Phosphate-phosphate buffer.

occurs, it can be calculated (Albert, 1962) that chlorhexidine exists 100% as the mono-cation and this dissociates further to the di-cation to the extent of 61%. At pH 7·0, where uptake reaches its maximum, 99·95% of the drug is still in the form of the mono-cation and 0·0009% as the di-cation. The decrease in the concentration of the mono-cation is only about 0·05%, thus for the adsorption of the mono-cation the change in the nature of the cell surface with pH is playing a greater role than that of the changing ionisation of the drug itself.

In experiments involving long contact periods with chlorhexidine, it was noted that the supernatant layer remained turbid on centrifugation and that the assay procedure of Holbrook (1958) was invalidated. This suggested that leakage of intracellular material might have occurred due to damage to the cytoplasmic membrane. Accordingly, the leakage of material absorbing at 260 mμ and of pentoses was investigated (Fig 3).

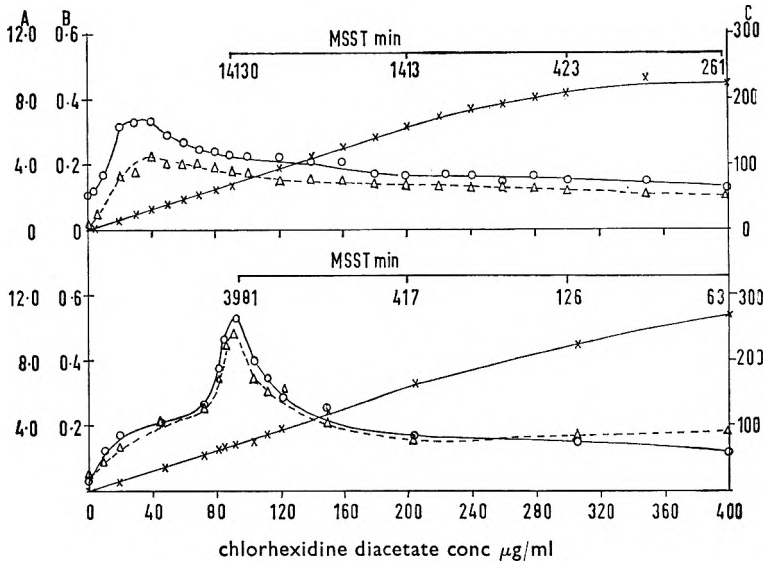

FIG. 3. Effect of concentration of chlorhexidine diacetate on a bacterial suspension 1·2 mg dry wt cells/ml in 0·013M phosphate buffer pH 7·3 after 6 hr at 20°. Upper figure *Staph. aureus*. Lower figure *E. coli*. ×——×, Adsorption of chlorhexidine diacetate μg/mg dry wt cells. ○——○, Leakage of 260 mμ absorbing material. △---△, Leakage of pentoses. Also shown are mean single survivor times (MSST of suspensions determined under identical conditions, see Table 1. A. Pentoses, μg/mg of dry cells. B. Extinction at 260 mμ/mg dry wt of cells. C. Uptake of chlorhexidine diacetate μg/mg of dry wt of cells.

A curve representing drug adsorption is superimposed. It is clear that chlorhexidine promotes the leakage of these two intracellular components and the effect of drug concentration on leakage shows a characteristic diphasic effect; the process of drug uptake proceeds in a continuous curve. This behaviour is similar to that found for cetyltrimethylammonium bromide and polymyxin (Newton, 1953) but contrasts with the result found for hexylresorcinol by Beckett, Robinson & Patki (1959) who found leakage and uptake to be both monophasic. A possible explanation for the decrease in leakage (the second phase of the leakage curves) may be that the leakage phenomenon is due, at least in part, to the activity of autolytic enzymes following chlorhexidine damage. These in higher concentrations of chlorhexidine may be inhibited. Alternatively, the cell surface may be sealed or the cytoplasmic membrane congealed, again preventing leakage.

It is possible, from available data, to calculate the approximate concentration at which a monolayer of drug molecules is formed on the surface of a bacterial cell. Thus, if it is assumed that *E. coli* is a cylinder $2{\cdot}0\,\mu \times 0{\cdot}8\,\mu$ surmounted at either end by hemispheres of radius $0{\cdot}25\,\mu$ then the surface area is $5{\cdot}812\,\mu^2 = 5{\cdot}812 \times 10^8$ Å^2. If it is assumed that the chlorhexidine molecule is orientated with one chlorine atom at the cell surface and the rest of the molecule perpendicular to the surface (a condition for optimal packing but unlikely to occur in fact) then the molecule will occupy an area of 26Å^2, thus the maximum number of molecules accommodated as a monolayer is $5{\cdot}812 \times 10^8/26 = 0{\cdot}224 \times 10^8$ molecules, or, from Avagadro's number, $1{\cdot}867 \times 10^{-8}\,\mu$g chlorhexidine (base) per cell. 1 mg dry wt cells of *E. coli* $= 4 \times 10^9$ cells thus $1{\cdot}867 \times 10^{-8} \times 4 \times 10^9 = 74{\cdot}68\,\mu$g base $= 85{\cdot}5\,\mu$g of the diacetate/mg dry wt of cells. The actual figure is probably much less than this. Thus it is possible that adsorption over and above this level might be due to the building up of multilayers of the drug which may be a feature of the leakage inhibition phenomenon, or, alternatively, to penetration of drug into the interior (cytoplasm) of the cell.

Further evidence about the relative roles of drug uptake and leakage of cell constituents may be obtained by studying the time course of the two processes.

Using a concentration of chlorhexidine which causes peak leakage (Fig. 4), shows that whereas the process of drug-uptake is rapid, the

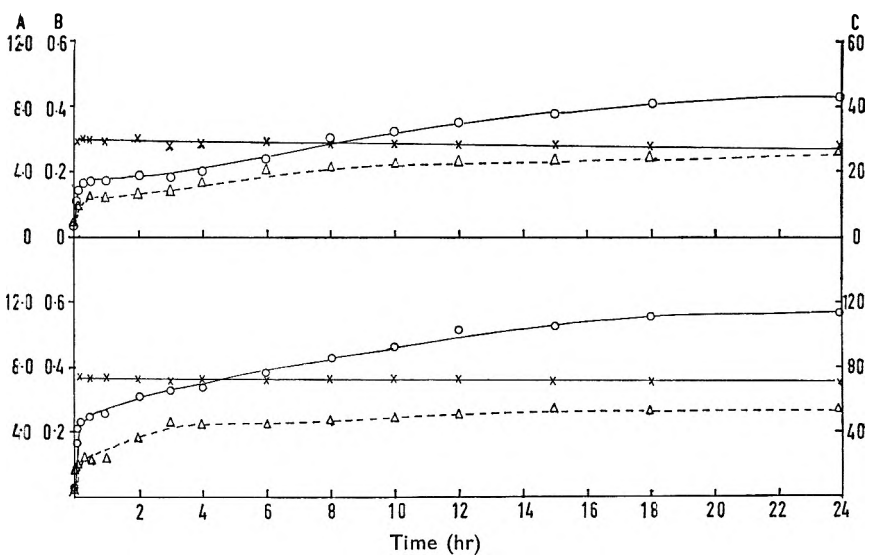

FIG. 4. ×——×, Rate of uptake of chlorhexidine diacetate by bacterial suspensions 1·2 mg dry wt cells/ml in 0·013M phosphate buffer pH 7·3 at 20°. Upper figure *Staph. aureus*. Lower figure *E. coli*. —○—, Leakage of 260 absorbing material. – – △ – – Leakage of pentoses. Concentration of chlorhexidine used for *E. coli* 93·6 μg/ml and for *Staph. aureus* 40 μg/ml. A. Pentoses, μg/ml dry wt of cells. B. Extinction at 260 mμ/mg dry wt of cells. C. Uptake of chlorhexidine diacetate μg/mg dry wt of cells.

leakage process increases with time. This may be due to a slow disintegration of the cytoplasmic membrane, or to the activation of enzymes which destroy the membrane.

Further evidence for cytolytic damage may sometimes be derived by studying extinction changes in bacterial suspensions. In Fig. 5 are

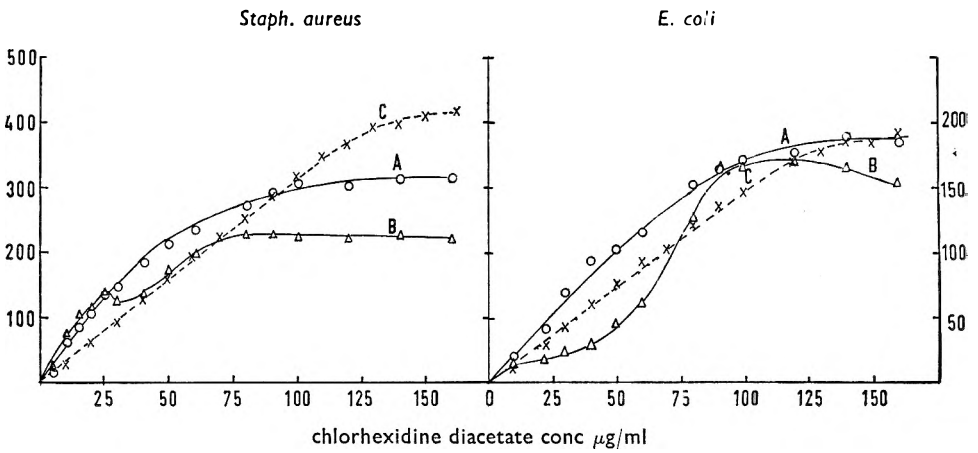

FIG. 5. ×---×, Adsorption of chlorhexidine diacetate by aqueous suspensions of organisms 0·6 mg dry wt cells/ml and extinction $E \times 10^3$ at 500 mμ ○—○ after 10 min and △—△ 180 min contact at 20°. Reference cell contains untreated bacterial suspension 0·6 mg dry wt cells/ml. R.h. ordinate, $E \times 10^3$. L.h. ordinate, uptake of chlorhexidine μg/mg dry wt of cells.

plotted extinction changes for *E. coli* after 10 min (curve A), 180 min (curve B) with the uptake (curve C) superimposed. A comparison of curves A and C suggest that the first effect of the drug is to cause a steady increase in turbidity which may be due to changes in the reflecting surface of the cells caused by adsorbed drug or similar changes due to a physical alteration of the surface. However, if the turbidities are re-measured after 180 min contact (curve B) a different picture is obtained. Over the range 0–90 μg/ml the turbidity is less, the fall (difference between A and B) being greatest over the range 40–60 μg/ml, corresponding to an adsorption 60–90 μg/mg dry wt of cells which, in turn, corresponds (Fig. 3) to the adsorption level at which peak leakage occurs. This suggests that this evidence of gross cytolytic damage supported by leakage is linked with a well-marked change in optical (reflecting properties) of the cell.

The fall in turbidity observed at concentrations greater than 110 μg/ml can be directly attributable to agglutination of the suspension which can be observed by eye after 180 min.

The data obtained for *Staph. aureus* (Fig. 5) lend themselves to similar interpretations although agreement between extinction differences after 10 and 180 min contact and the peak leakage phenomenon are not as exact.

Evidence obtained so far, suggests that chlorhexidine behaves in many ways like cationic antibacterial agents with surface-active properties such as, for example, cetyltrimethylammonium bromide and polymyxin; consequently the effect of the drug on the air/water interface and the interfacial tension between water and normal hexane were determined using the micrometer syringe drop volume method (Fig. 6). Little change

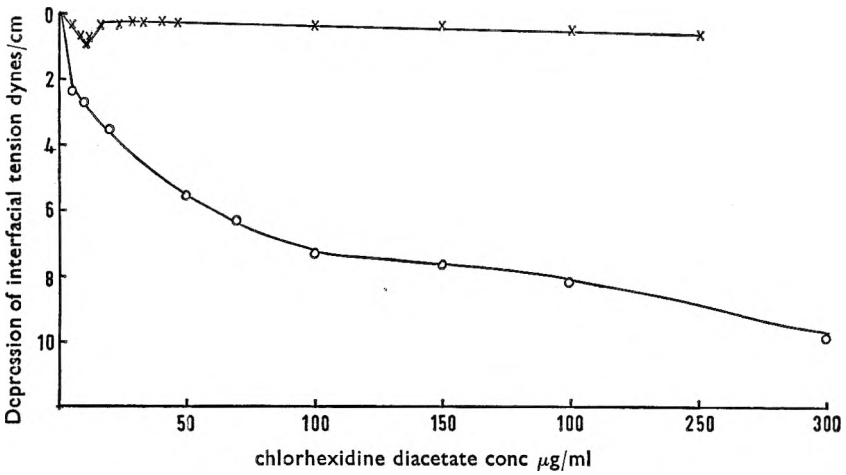

FIG. 6. Interfacial tensions of chlorhexidine diacetate in water at 20°. ×——×, air/water interface (interfacial tension 72·4 dynes/cm). ○——○, n-hexane/water interface (interfacial tension 51·0 dynes/cm).

was observed in the air/water interface but the interfacial tension between water and n-hexane falls progressively. Polymyxin shows a similar behaviour (Few & Schulman, 1953).

TABLE 1. EFFECT OF CONCENTRATION OF CHLORHEXIDINE DIACETATE ON THE MEAN SINGLE SURVIVOR TIME (MSST) OF SUSPENSIONS OF BACTERIA 1·2 MG DRY WT/ML IN 0·013 M PHOSPHATE BUFFER pH 7·3 AT 20°

Concn. chlorhexidine diacetate μg/ml	log concn.	*E. coli*		*Staph. aureus*	
		MSST min	log MSST	MSST min	log MSST
800	2·9031			40·5	1·6075
600	2·7782	19 ·	1·2788	89	1·9494
500	2·6990	33	1·5185	174	2·2405
400	2·6021	63	1·7993	261	2·4166
300	2·4711	126·5	2·1021	423	2·626
*200	2·3010	417	2·62	1,413	3·15
*90	1·9542	3,981	3·6	14,130	4·15

* Derived by extrapolation.

Table 1 shows the relation between the mean single survivor time (MSST) and chlorhexidine concentration. There is a linear response between log MSST and log dose and by extrapolation an indication of the order of the MSST for lower concentrations of chlorhexidine can be deduced.

Discussion

The main findings to date are summarised in Fig. 3 where drug adsorption, leakage of cell constituents and mean single survivor times are shown. If it is assumed that mean single survivor times are a reliable estimate of the bactericidal efficiency of a system of this nature, where there is a tendency for agglutination to occur (Berry & Bean, 1954), it can be seen that there is no obvious relationship between the amount of cell constituents released and the number of organisms killed. Few & Schulman (1953) using polymyxin and Salton (1951) using cetyltrimethylammonium bromide found that for concentrations of drug which kill less than a certain percentage of cells there was a linear relationship between the proportion of cells killed and the amount of 260 mμ absorbing material released. Such a relationship may exist in concentrations of chlorhexidine lower than that causing maximum leakage but, over the range of concentrations studied, no such relationship holds.

Treatment with all concentrations of chlorhexidine causes a level of leakage greater than that occurring in untreated cells but with high drug concentrations it would appear as though the adsorbed drug is exerting a secondary effect.

It may be that the mode of action of the drug is to react with the cell causing a disorientation of the lipoprotein membrane, by virtue of the lipophilic groups of the drug molecule, so that the membrane no longer fulfils its function as an osmotic barrier (Gale, 1963). Once this initial reaction has occurred the subsequent events depend upon the concentration of drug present which may prevent leakage from the damaged cell by physical sealing in, due to formation of a complete layer or layers of drug on the cell surface, a possibility indicated by the calculation presented, or by inactivation of autolytic enzymes or by denaturation of the cytoplasmic membrane or cytoplasm. On the other hand it may be that a secondary and more profound lethal effect occurs with high dose levels of chlorhexidine.

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Antibacterial properties of 5-nitro-2-furylglyoxylidene derivatives

W. R. BUCKETT* AND D. KIDD

5-Nitro-2-furylglyoxal has been prepared and nine new derivatives synthesised. These showed similar *in vitro* antibacterial activity to the corresponding 5-nitrofurfurylidene analogues. The antibacterial activity of the 5-nitro-2-furylglyoxylidenes could not be demonstrated in the serum or urine of rats after oral administration.

MANY diverse structures have been based upon the 5-nitrofuran molecule following the original work of Dodd & Stillman (1944), which led to the development and use of nitrofurazone (5-nitrofurfuraldehyde semicarbazone) (Dodd, 1946) as an effective antibacterial agent. In view of the success of the nitrofurans in a variety of clinical infective conditions, the 5-nitro-2-furylglyoxylidene derivatives were considered to be worth examining as potential antibacterial drugs.

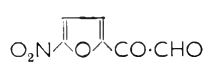
Soldabols & Hillers (1960) obtained the dihydrate of 5-nitro-2-furyl glyoxal, and Gualtieri, Riccieri & Stein (1962) demonstrated its reactivity by preparing several unstable aromatic glyoxylidene derivatives. Later Caradonna, Gualtieri & Riccieri (1962) demonstrated the bacteriostatic activity of 5-nitro-2-furylglyoxal monosemicarbazone *in vitro*. We obtained 5-nitro-2-furylglyoxal as an oil by oxidation of 2-acetyl-5-nitrofuran (Hayes & O'Keefe, 1954) with selenium dioxide in aqueous acetic acid. From this were prepared derivatives of 1-aminohydantoin and 3-amino-2-oxazolidone, the latter being found to possess high antibacterial activity *in vitro*. In view of this high activity, the series of derivatives of 5-nitro-2-furylglyoxal was extended and compared with analogous derivatives of 5-nitrofurfuraldehyde for antibacterial activity *in vitro* and acute toxicity in mice.

Experimental

CHEMICAL

All melting-points are uncorrected. Yields are based on 5-nitro-2-furylglyoxal dihydrate.

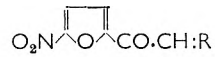
5-*Nitro*-2-*furylglyoxal* (I). 2-Acetyl-5-nitrofuran (Hayes & O'Keefe, 1954) (40 g) in acetic acid (110 ml) was heated under reflux with selenium dioxide (28·4 g in 10 ml water) for 3 hr. The hot reaction mixture was then filtered (kieselguhr) to remove metallic selenium, and concentrated *in vacuo*. The *glyoxal* was obtained as a dark red oil (26·1 g, 45%).

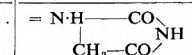
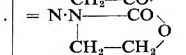
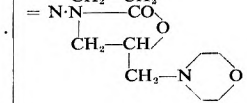
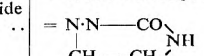
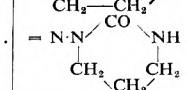


(I)

From The Research Department, Edinburgh Pharmaceutical Industries Limited, Wheatfield Road, Edinburgh, 11. * Present address: Pharmacology Research Department, Organon Laboratories Limited, Newhouse, Lanarkshire, Scotland.

TABLE 1. 5-NITRO-2-FURYLGLYOXYLIDENE DERIVATIVES



Derivative No.	Parent compound	R	Recrystallisation solvent	% yield	m.p. °	Formula	Found %					Required %				
							C	H	Cl	I	N	C	H	Cl	I	N
II	1-Aminohydantoin		Ethanol	40	241	$C_9H_6N_4O_6$	40·4	2·5			20·9	40·6	2·3			21·0
III	3-Amino-2-oxazolidone ..		Acetic acid	82	230	$C_9H_7N_3O_6$	43·0	2·9			16·5	42·7	2·8			16·6
IV	3-Amino-5-morpholinomethyl-2-oxazolidone ..		Nitroethane	57	213	$C_{14}H_{16}N_4O_7$	47·9	4·8			15·8	47·7	4·6			15·9
V	IV as hydrochloride		—	—	235–240	$C_{14}H_{17}N_4O_7Cl$			9·1					9·1		
VI	IV as methiodide		Aqueous ethanol	—	232–235	$C_{15}H_{19}IN_4O_7$	37·5	4·1		25·9	11·3	36·4	3·9		25·7	11·3
VII	*p*-Hydroxybenzhydrazide ..		Nitroethane	70	248	$C_{13}H_9N_3O_6$	50·7	3·0			13·6	51·5	3·0			13·9
VIII	(Hydrazinoformylmethyl)-trimethylammonium chloride	= N·NH·CO·CH$_2$·N$^+$Me$_3$Cl$^-$	Water/ethanol	66	242	$C_{11}H_{15}N_4O_5Cl$	41·2	4·5	11·2		17·8	41·2	4·7	11·1		17·6
IX	1-Amino-2-imidazolidone ..		Dimethylformamide	86	251	$C_9H_8N_4O_5$	43·0	3·2			22·0	42·9	3·2			22·2
X	1-Aminotetrahydro-2-pyrimidone		Nitroethane	60	261	$C_{10}H_{10}N_4O_5$	45·3	4·0			21·1	45·1	3·8			21·1

Derivatives of 5-Nitro-2-furylglyoxal (II–X; Table 1). The general preparative method consisted of reacting the glyoxal with the amine or amine salt in ethanol or aqueous ethanol, and is illustrated by the following preparation of two of the products.

(*a*) 5-Nitro-2-furylglyoxal (1·0 g) in ethanol (10 ml) was heated with 1-aminohydantoin hydrochloride (Jack, 1959) (0·6 g) in boiling water (2·0 ml) for 15 min. The *product* (II) was filtered, dried and twice crystallised (ethanol) to yield fine pale yellow needles (0·65 g, 40%) m.p. 238–241° (decomp.) ν_{max} 3400, 3300 (NH), 1740 (C : O), 1645 (CO·NH), 1580 (C : N), 1515 and 1345 cm^{-1} (NO_2).

(*b*) To a warm solution of 5-nitro-2-furylglyoxal (12·7 g) in ethanol (60 ml) was added, with mixing, a warm solution of 3-amino-5-morpholinomethyl-2-oxazolidone (13·2 g) (Gever, 1957) in ethanol (70 ml). After heating under reflux for 1 hr the reaction mixture was cooled, and the *product* (IV) collected. Recrystallisation from nitroethane gave yellow blades (12·5 g, 57·3%), m.p. 214–215°, ν_{max} 3200 (NH), 1780 (C:O), 1650 (CO·NH), 1580 (C:N), 1510 and 1340 cm^{-1} (NO_2).

Derivatives of 5-nitrofurfuraldehyde (XI–XIX, Table 2). These were prepared according to literature methods as follows: the derivatives of 1-aminohydantoin (XI) (Jack, 1959), of 3-amino-2-oxazolidone (XII) (Gever, O'Keefe, Drake, Ebetino, Michels & Hayes, 1955), of 3-amino-5-morpholinomethyl-2-oxazolidone (XIII) and of its hydrochloride (XIV) and methiodide (XV) (Gever, 1957), of *p*-hydroxybenzhydrazide (XVI) (Carron, Jullien, Julia & Garczynska, 1963), of hydrazinoformylmethyl)-trimethylammonium chloride (XVII) (Ward, 1953), of 1-amino-2-imidazolidone (XVIII) (Michels & Gever, 1956) and of 1-aminotetrahydro-2-pyrimidone (XIX) (Michels, 1960).

BIOLOGICAL

Bacteriostatic activity. Compounds were dissolved in distilled water or acetone purified by distillation over potassium permanganate. The solutions were serially diluted by 2-fold steps in nutrient broth (Oxoid No. 2–CM 67) and each tube was inoculated with 0·1 ml of an 18 hr culture of one of the following organisms:

Staphylococcus aureus (benzylpenicillin-resistant), *Escherichia coli*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Proteus vulgaris* (all isolated and identified at St. Luke's Hospital, Bradford) and *Bacillus subtilis* (NCTC 6276). Determinations using *Salmonella typhimurium* (Strain 305, Allen & Hanburys Ltd., Veterinary Department), *Salmonella dublin* (Strain 98, Allen & Hanburys Ltd., Veterinary Department) and *Streptococcus faecalis* (E186, PHLS (Strep. R.L.)) were made in glucose-peptone water. Each tube was then incubated at 37° for 24 hr, and the minimal inhibitory concentrations (MIC) were determined as the lowest concentrations of compounds which prevented growth visible to the naked eye.

TABLE 2. A COMPARISON OF THE MINIMUM INHIBITORY CONCENTRATIONS AND ACUTE TOXICITY OF DERIVATIVES OF GLYOXAL AND FURFURALDEHYDE

Parent compound	Derivative No.	MIC μg/ml (24 hr)									LD50 mg/kg in mice (24 hr)	
		Staph. aureus	*B. subtilis*	*Pr. vulgaris*	*Ps. aeruginosa*	*E. coli*	*Kl. aerogenes*	*Salm. typhimur.*	*Salm. dublin*	*Strep. faecalis*	*Oral*	*Intra-peritoneal*
5-Nitro-2-furylglyoxylidene derivatives												
1-Aminohydantoin	II	128	64	256	—	512	512				>1000	110 (90–120)
3-Amino-2-oxazolidone	III	1	4	8	—	8	8	2	3	1	>1000	88 (86–91)
3-Amino-5-morpholinomethyl-2-oxazolidone	IV	8	4	64	—	16	64				300–500	64 (50–81)
IV hydrochloride	V	16	16	132	—	64	64				300–400	65 (60–71)
V methiodide	VI	>256 <1000	64	>256 <1000	—	>256 <1000	>256 <1000				>1000	390 (350–430)
p-Hydroxybenzhydrazide	VII	256	>256 <1000	>256 <1000	>256 <1000	>256 <1000	>256 <1000				>1000	>1000
(Hydrazinoformylmethyl-)trimethyl-ammonium chloride	VIII	64	32	64	—	128	64				>1000	470 (400–560)
1-Amino-2-imidazolidone	IX	2	8	32	—	16	8	3	2	4	>1000	410 (340–470)
1-Aminotetrahydro-2-pyrimidone	X	32	128	32	—	64	128				>1000	1100 (780–1400)
5-Nitrofurfurylidene derivatives												
1-Aminohydantoin	XI†	64	32	128	—	16	16				>1000	110 (100–130)
3-Amino-2-oxazolidone	XII*	4	4	16	—	2	1	2	1	2	>1000	1600 (1400–1800)
3-Amino-5-morpholinomethyl-2-oxazolidone	XIII	8	4	32	—	4	16				>1000	1000
IV hydrochloride	XIV	8	4	128	—	16	16				≤1000	190 (150–240)
V methiodide	XV	>256 <1000	64	>256 <1000	—	>256 <1000	>256 <1000				>1000	430 (380–490)
p-Hydroxybenzhydrazide	XVI	>256 <1000	8	>256 <1000	>256 <1000	>256 <1000	256				>1000	1000
(Hydrazinoformylmethyl-)trimethyl-ammonium chloride	XVII	128	128	256	—	128	256				>1000	440 (400–480)
1-Amino-2-imidazolidone	XVIII	4	4	64	—	8	4				>1000	1000
1-Aminotetrahydro-2-pyrimidone	XIX	8	8	4	—	16	64				600	720 (500–1000)

* Furazolidone. † Nitrofurantoin.

The effect of serum on the antibacterial activity of compounds was determined by estimating the MIC using nutrient broth containing 25 or 50% horse serum.

Bactericidal activity. Bactericidal tests were carried out by subculturing from tubes not showing growth in the bacteriostatic tests, onto nutrient agar (Oxoid Blood Agar No. 2–CM271). After overnight incubation the presence or absence of growth was noted.

Bacteriostatic activity of serum and urine in rats. Groups of four albino rats (60–80 g) were dosed orally with 1 g/kg of the compound suspended in 1% methylcellulose. Control groups received 1% methylcellulose alone. Blood and urine samples were taken 1 and 2 hr later. The blood was allowed to clot, centrifuged and serum removed. 0·1 ml samples of serum and urine were then placed in 5 mm diameter wells in agar plates seeded with *Staph. aureus*, *E. coli* or *Kl. aerogenes*. The plates were refrigerated at 4° for 90 min, then incubated overnight at 37° and examined for growth inhibitory zones around the wells.

Acute toxicity in mice. Albino male mice (20–25 g) were used and randomised into groups of five. Compounds were standardised for particle size between 100- and 200-mesh sieves and the resultant powder suspended in distilled water for oral administration or in normal saline for intraperitoneal injection using 1% methylcellulose as suspending agent. After administering the compounds in a dose volume of 0·2 ml/20g mouse, the percentage mortality was recorded after 24 hr and 5 days. The LD50 was computed by the method of Litchfield & Wilcoxon (1949).

Results

Antibacterial activity in vitro. The minimum inhibitory concentrations of the compounds tested against a number of organisms are shown in Table 2.

Subcultures taken at 24 hr from the bacteriostatic test samples showed that only the 1-aminotetrahydro-2-pyrimidone derivatives were bactericidal at concentrations within one or two tubes of the MIC. The other derivatives only demonstrated bactericidal activity at higher concentrations.

TABLE 3. THE EFFECT OF SERUM ON THE BACTERIOSTATIC ACTIVITY OF THREE NITROFURAN DERIVATIVES

No.	Derivative	Concentration of horse serum %	MIC μg/ml (24 hr)				
			Staph. aureus	*B. subtilis*	*Pr. vulgaris*	*E. coli*	*Kl. aerogenes*
XII	3-(5-nitrofurfurylidene-amino)-2-oxazolidone (furazolidone)	0	4	4	16	2	1
		25	32	32	64	16	32
		50	64	64	128	32	32
III	3-(5-nitro-2-furylglyoxyl-ideneamino)-2-oxazolidone	0	1	4	8	8	8
		25	16	16	32	32	32
		50	32	32	64	64	64
IX	1-(5-nitro-2-furyl-glyoxylidene amino)-2-imidazolidone	0	2	8	32	16	8
		25	32	32	128	128	64
		50	64	128	128	128	128

With the three derivatives (III, IX and XII) tested in the presence of horse serum, it was found that in a 50% serum concentration, bacteriostatic activity was markedly reduced (Table 3). In several instances, a 25% serum concentration had the same effect.

TABLE 4. THE BACTERIOSTATIC EFFECT OF URINE AND SERUM FROM RATS TESTED ORALLY WITH NITROFURAN COMPOUNDS

No.	Derivative	Bacteriostatic activity 1 hr after oral administration					
		Serum			Urine		
		Staph. aureus	*E. coli*	*Kl. aerogenes*	*Staph. aureus*	*E. coli*	*Kl. aerogenes*
III	3-(5-Nitro-2-furylglyoxylidene-amino)-2-oxazolidone	O	O	+	O	O	O
XII*	3-(5-nitrofurfurylideneamino-)2-oxazolidone	O	+	+	O	+	+
IX	1-(5-Nitro-2-furylglyoxylidene-amino)-2-imidazolidone	O	O	O	O	O	O
XVIII	1-(5-Nitrofurfurylideneamino)-2-imidazolidone	O	+	+	+	+	+
X	Tetrahydro-1-(5-nitro-2-furylgly-oxylideneamino)-2-pyrimidone	O	O	O	O	O	O
XIX	Tetrahydro-1-(5-nitrofurfurylidene-amino)-2-pyrimidone	O	O	O	+	+	+
	Control 1% methylcellulose	O	O	O	O	O	O

O = Inactive. + = Bacteriostatic activity. * Furazolidone.

Bacteriostatic effect in rat serum and urine. Table 4 shows the bacteriostatic effect of three 2-furylglyoxylidene and the three corresponding furfurylidene derivatives in the serum and urine of rats 1 hr after oral administration of 1 g/kg of compound suspended in 1% methylcellulose in distilled water.

Acute toxicity in mice. The 24-hr LD50 values in mg/kg are shown in Table 2. After 5 days the mortality did not alter in either the orally or intraperitoneally dosed groups. Intraperitoneally, the 2-furylglyoxylidene derivatives were more toxic than the furfurylidene derivatives in most instances. The 1-aminohydantoin and 1-aminotetrahydro-2-pyrimidone derivatives were similarly toxic, as were the quaternary derivatives. The derivatives of *p*-hydroxybenzhydrazide possessed very low toxicity. Orally, both the 2-furylglyoxylidene and furfurylidene series exhibited low acute toxicity in mice, but the 2-furylglyoxylidene derivative of 3-amino-5-morpholinomethyl-2-oxazolidone and its hydrochloride, and the furfurylidene derivative of 1-aminotetrahydro-2-pyrimidone were more toxic. In all instances the urine was a yellow to red colour, and sedation and respiratory depression preceded death.

Discussion

In the 2-furylglyoxylidene and furfurylidene series the minimal inhibitory concentrations *in vitro* were about the same. The resistance of *E. coli* and *Kleb. aerogenes* to 1-(5-nitro-2-furylglyoxylideneamino)-hydantoin was anomalous in this respect. Only moderate activity

against some organisms was found when three pairs of compounds were tested *in vivo* in the rat (Table 4). These findings partially agree with the report (Interscience Conference, 1963) claiming good results with 1-(5-nitrofurfurylideneamino)-2-imidazolidone in animals and man. Even the large oral dosage of 1 g/kg in the rat did not give blood levels sufficiently high to inhibit the growth of *Staph. aureus*, and activity against *Ps. aeruginosa*, even *in vitro*, was not found. No activity was found in the serum of rats treated with tetrahydro-1-(5-nitrofurfurylideneamino)-2-pyrimidone. The 2-furylglyoxylidene analogues showed no activity in either the urine or the serum. This may be due to their breakdown in the animal body to give inactive metabolites in contrast tc the furfurylidenes, which may be active themselves *in vivo* or may be broken down to give metabolites possessing antibacterial activity.

Acknowledgements. Acknowledgement is made to Dr. W. A. Cox for work with *Salm. typhimurium*, *Salm. dublin* and *Strept. faecalis*, and to Mrs. T. P. S. Tweedie and Mr. A. Harrison for technical assistance.

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Oxidation of benzaldehyde in some multiphase betaine-benzaldehyde-water systems

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The results of oxidation studies in ternary multiphase betaine-benzaldehyde-water systems show that the maximum rate of oxidation depends primarily on the benzaldehyde content and composition of the phase with the shortest induction period in the system under examination. The effects of chain length and concentration of the S-amphiphile (betaine) are important only in so far as they influence the phase equilibria of the ternary systems.

IN attempting to establish the relation between aldehyde concentration and oxidation rate in multiphase systems it is essential to know both the number and proportion of phases present and the amount of aldehyde in each. The number of phases in the dispersions prepared for oxidation studies was known from the phase equilibrium diagrams determined by Swarbrick & Carless (1963). The proportion of each phase and the concentration of benzaldehyde therein was readily calculated from a knowledge of the length and direction, respectively, of the relevant tie line (Swarbrick, 1963).

Experimental

The experimental details and the nomenclature used are as previously described (Swarbrick & Carless, 1964).

Results

OXIDATION IN $L_1 + L_2$ SYSTEMS

The maximum rate of oxidation in these systems was generally reached within 30 min of preparation. At the higher benzaldehyde concentrations, the induction period was often absent and the initial rate was the maximum one. The oxygen absorption in these systems is therefore dependent on the concentration of aldehyde in the L_2 phase and independent of that in the L_1 phase, which has an induction period of at least 10 hr.

The rates/litre of *binary* systems containing increasing amounts of benzaldehyde in water are shown in Fig. 1. These suspensions were extremely unstable (physically), the drops of the dispersed L_2 phase coalescing within a few minutes of being prepared. When account is taken of the concentration of the aldehyde in the L_1 phase, the rate/litre is directly related to the concentration of aldehyde in the dispersed L_2 phase. The calculated mean rate/g is 6·6 ml O_2/hr/g benzaldehyde in the L_2 phase.

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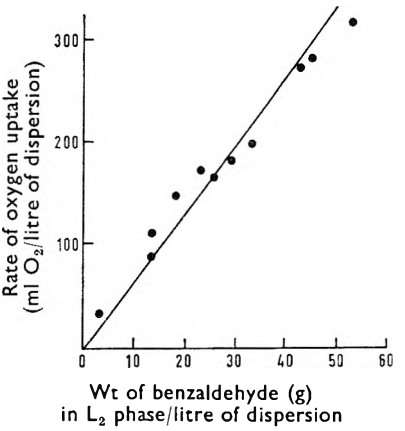

FIG. 1. Dependence of the rate of oxygen absorption per litre of dispersion on the concentration of benzaldehyde in the L_2 phase of *binary* $L_1 + L_2$ systems.

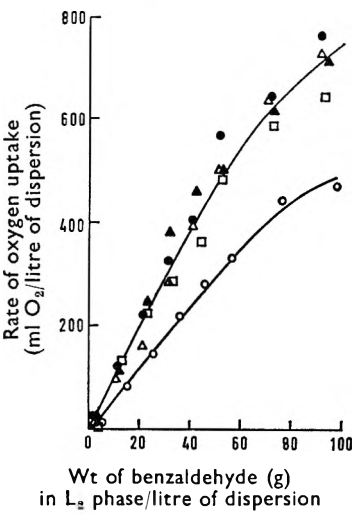

FIG. 2. Dependence of the rate of oxygen absorption per litre of dispersion on the concentration of benzaldehyde in the L_2 phase of $L_1 + L_2$ systems. Betaine concentration: 0·01M. ○, C_{10}; ●, C_{11}; △, C_{12}; ▲, C_{14}; □C_{16}.

The rate of oxidation of different concentrations of benzaldehyde dispersed in equimolar concentrations of five betaine homologues is shown in Fig. 2. The rates/litre in the C_{11}, C_{12}, C_{14} and C_{16} ternary systems are similar for equivalent concentrations of aldehyde in the L_2 phase, the mean rate/g over the linear portion being 10·5 ml O_2/hr/g benzaldehyde in the L_2 phase. In the C_{10} system the mean rate is lower at 6·3 ml O_2/hr. From the point of view of physical stability these dispersions resembled the binary suspensions of benzaldehyde in water, in that the drops of dispersed phase coalesced and pooled rapidly. The

emulsified dispersions prepared with the higher betaine homologues were however stable until the concentration of aldehyde in the L_2 phase exceeded 40 to 50 g/litre of dispersion.

The effect of betaine concentration on the rate of oxygen uptake was also investigated, typical results being presented in Fig. 3. The rate/g

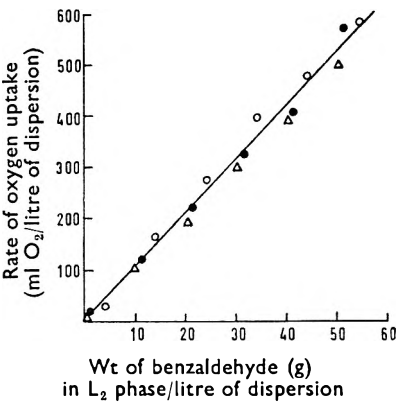

FIG. 3. Dependence of the rate of oxygen absorption per litre of dispersion on the concentration of benzaldehyde in the L_2 phase of $L_1 + L_2$ systems containing different concentrations of betaine C_{11} (% w/w). Betaine C_{11}: ○, 0·12; ●, 0·24; △, 0·35.

of 10·5 ml O_2/hr/g benzaldehyde in the L_2 phase is identical to that found earlier and independent of the betaine concentration over the range studied.

OXIDATION IN L_1 + LC SYSTEMS

The induction period of the L_1 + LC systems lay between 4 and 6 hr. This is slightly less than that found in the LC systems alone (Swarbrick & Carless, 1964). This induction period is also less than that in L_1 systems and implies that the maximum rates of oxidation are mainly due to the benzaldehyde present in the LC phase.

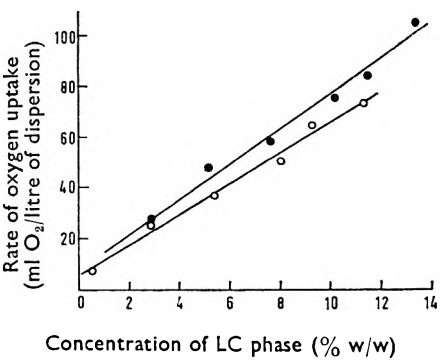

FIG. 4. Dependence of the rate of oxygen absorption per litre of dispersion on the concentration of the LC phase, of fixed composition, in the L_1 + LC phase region of the C_{12} system. Composition of the LC phase (% w/w): ○, benzaldehyde 41·9; betaine C_{12} 23·9: ●, benzaldehyde 42·5; betaine C_{12} 23·9.

The influence of benzaldehyde concentration on the oxidation rate was studied in C_{12} and C_{14} systems prepared along known tie lines. Consequently, the composition of the LC phase remained constant in any one series of dispersions. Typical results (Fig. 4) show that the rate of oxidation per litre in two different series of dispersions is dependent on the percentage w/w of LC present. The higher rates are due to an LC phase containing a higher proportion of benzaldehyde. Instead of passing through the origin, the extrapolated lines cut the ordinate at a point equivalent to 7 ml O_2/hr/litre of dispersion. This rate is caused by background oxidation of the L_1 phase. The agreement between the mean rates/g on a molar ratio basis (Table 1) is reasonable, especially in view of the assumptions made about the concentration and composition of the LC phases present in these dispersions (Swarbrick & Carless, 1963).

TABLE 1. OXIDATION OF BENZALDEHYDE IN L_1 + LC SYSTEMS AT 25°

Ternary system	Benzaldehyde concentration in LC phase % w/w	Molar ratio of benzaldehyde to betaine	Rate/litre due to LC	Rate/g due to LC
C_{12}	42·5	4·84	72·0	1·73
,, ...	41·9	4·77	60·0	1·43
C_{14}	21·9	3·09	20·0	0·91
,, ...	21·3	3·02	17·0	0·80

These results contrast with the rate/g of the C_{14} system containing 100% of LC (0·23 ml O_2/hr), and suggest that although a linear relationship is obtained over a range of concentrations, the rate/g falls as the concentration of LC is increased further in these systems.

OXIDATION IN L_1 + L_2 + LC SYSTEMS

Depending on the aldehyde concentration, steady state conditions were attained in 140 min or less. As before, the induction period was reduced as the aldehyde concentration increased and was, on occasions, absent. The times indicate that the maximum rate of oxidation is largely due to the benzaldehyde in the dispersed L_2 phase.

Dispersions were prepared lying along a series of lines running parallel to the base line of the ternary L_1 + L_2 + LC triangle. In this way the concentration of LC, in any one series of dispersions, remained constant and could be readily calculated together with the concentration of the L_2 phase. The composition of each conjugate phase is invariant (Swarbrick & Carless, 1963). The results for the six C_{14} series investigated are presented in Fig. 5. The rate/litre increases directly with the concentration of the L_2 phase, with a low level of background oxidation arising presumably from the LC phase. The mean rate/g is 3·7 O_2/hr/g aldehyde in the L_2 phase, the same rate being obtained in four similar series of C_{12} systems. This value is considerably lower than that in the L_1 + L_2 systems in which the observed rate of oxidation is also presumed to be dependent on the amount of aldehyde in the L_2 phase.

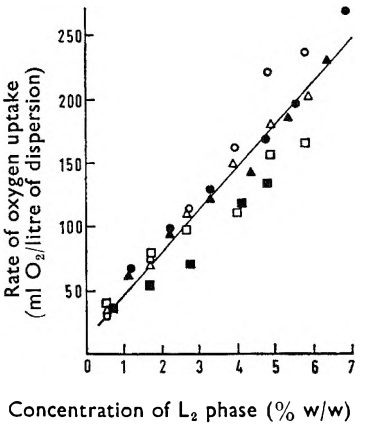

FIG. 5. Dependence of the rate of oxygen absorption per litre of dispersion on the concentration of benzaldehyde in the L_2 phase of $L_1 + L_2 +$ LC systems containing different concentrations of an LC phase (composition 21·9% w/w benzaldehyde, 21·1% w/w betaine C_{14}). Concentration of LC present (% w/w): ○, 3·22; ●, 5·59; △, 7·95; ▲, 10·36; □, 12·27; ■, 17·59.

Discussion

In the $L_1 + L_2$ systems the induction period is very short, and the rate of oxidation due entirely to the aldehyde in the dispersed droplets of the L_2 phase. These droplets are comparable to a bulk phase, containing a high concentration of benzaldehyde within which free movement of the molecules takes place. Propagation of the chain reaction is not therefore restricted, as was the case in the L_1 systems (Swarbrick & Carless, 1964), and the rate of oxidation depends on the rate of initiation. The high dependence of the rate, in these systems, on the catalyst concentration (Swarbrick, 1963) supports this view.

With the exception of the C_{10} betaine, the use of different betaine homologues has no effect on the rate at which benzaldehyde absorbs oxygen, indicating that possible changes in the state of the interface brought about in this way are unimportant. The rate is also unaffected by changes in betaine concentration. However, the rate/g falls, in all the systems, as the concentration of benzaldehyde is increased to above 40–50 g in the L_2 phase/litre of dispersion. Evidence has been obtained (Swarbrick, 1963) suggesting that this effect is caused not by insufficient catalyst but by a decrease in the specific interfacial area of the dispersed L_2 phase as a result of increased physical instability of the dispersions. Thus when the interfacial area falls sufficiently the rate of oxidation becomes dependent on the diffusion of oxygen from the continuous L_1 phase into the dispersed L_2 phase. In addition, initiation by water-soluble metal catalysts most probably occurs at this interface, and consequently the rate of initiation may also be reduced when the specific interfacial area falls below a certain value.

The above also accounts for the low rates of oxidation in the binary benzaldehyde-water systems and the ternary C_{10} systems. In the former the dispersions are unstable because of the absence of an S-amphiphile to provide an interfacial film, whereas in the latter the poor stability is due to the hydrocarbon chain of the C_{10} betaine being too short to confer the compound with reasonable emulsifying properties. The rate/g in the C_{10} system is therefore similar to that in the suspensions but well below that in the stable dispersions possessing high interfacial areas.

In the LC phase of L_1 + LC systems the rate/g increases with the molar ratio of benzaldehyde to betaine in a manner comparable to that in the L_1 systems. The rate/g of oxidation in the LC phase in these systems is higher than that in systems containing the LC phase alone. This indicates a reduction in the rate as the concentration of LC increases. This fall in rate is probably due to a reduction in the number of aldehyde molecules/g of the LC phase in contact with the L_1 phase as the concentration of LC is increased. An analogous situation is the reduction in specific surface area of solids as the mean particle size is increased. This concept is supported by the work of Honn, Bezman & Daubert (1951) who found that when the amount of oil deposited on silica gels exceeded that required to form a monolayer, a multilayer was formed in which only the outermost molecules remained immediately accessible to oxygen. As a consequence the oxidation rate began to decrease at this point, even though the oil molecules were in close contact with one another.

Although the induction period in the L_1 + L_2 + LC systems suggests that the oxygen uptake is mainly due to the aldehyde in the L_2 phase, the calculated rate/g of 3·7 ml O_2/hr is approximately a third of that recorded for the L_2 phase of the stable L_1 + L_2 dispersions and is even less than that found in the suspensions of benzaldehyde in water.

This low rate is very probably associated with the presence of the LC phase since this is the only phase difference between these systems. In the L_1 + L_2 − LC systems some of the drops of the L_2 phase are dispersed in the LC phase as well as in the continuous L_1 phase; this contrasts with the L_1 + L_2 systems in which all the L_2 drops are dispersed throughout the L_1 phase. These embedded drops of L_2, presumably dependent on the diffusion of oxygen through the highly viscous LC phase, will therefore have a longer induction period and a lower rate/g than the rest of the L_2 phase. Consequently, calculation of the true rate/g is complicated, since the maximum steady rate/litre, which was reached after approximately 2 hr, will be the sum of the oxygen uptake of benzaldehyde in two L_2 components having different induction periods. Presumably the LC phase at very high concentrations will replace the L_1 phase as the continuous phase and the rate will then approach that observed for the LC phase alone. It is likely therefore that the rate of oxidation in this triphasic region will depend to a large extent on where within the region a dispersion is prepared. It is also possible that changes in rate will occur when inversion of the triphasic "emulsion" leads to a change in the continuous phase.

All these results support the contention that a knowledge of the relevant portions of the phase equilibrium diagrams of the ternary systems under examination is necessary in order to relate the rate of oxidation to the concentration of aldehyde present in multiphase systems. Furthermore, since the maximum rate of oxidation is related very largely to the concentration of aldehyde present in only one phase, the use of a saturation ratio, which expresses the concentration of one phase in terms of another phase, is unnecessary when attempting to establish and explain oxidation rate/aldehyde concentration relationships.

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J. Pharm. Pharmacol., 1964, **16**, 677–689 Received April 30, 1964

Hypoglycaemic agents*: variants of tolbutamide

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Further new variants of tolbutamide (and two new imidazolines) are described.

THE preparation of further new variants of the active compounds, 1-butyl-3-toluene-*p*-sulphonylurea (I; R=Me, R′=Bu), 1-perhydroazepin-1′-yl-3-toluene-*p*-sulphonylurea [I; R=Me, R′=$C_6H_{12}N$], (compare Dulin, Oster & McMahon, 1961), and 1-cyclohexyl-3-(*p*-vinylbenzenesulphonyl)-urea (I; R=CH:CH_2, R′=cyclohexyl) (Hayman, Petrow & Stephenson, 1964), is described herein.

Arylsulphonylureas containing a secondary or tertiary alkyl group R′, (I), were prepared by reaction of the sulphonylurethane (II; R=Cl or Me, R′=OEt) with the appropriate amine (Table 1). Additionally, two water-soluble derivatives [II; R=Cl or Me; R′=$N(CH_2{\cdot}CH_2OH)_2$] were obtained by heating the urethanes with di(2-hydroxyethyl)amine.

p-Aminoacetophenone was converted into *p*-acetylbenzenesulphonyl chloride (III; R=CO·Me, R′=Cl) *via* the diazonium chloride (cf. Petrow, Stephenson & Wild, 1960), and thence to the sulphonylurea (I; R=CO·Me, R′=Bu) (Table 1). Whilst this work was in progress, the hypoglycaemic activity of this latter compound and of its *n*-propyl homologue, was reported by Blank, Farrina, Kerwin & Saunders (1961). Welles, Root & Anderson (1961) have discussed the metabolism of the cyclohexyl derivative (I; R=CO·Me, R′=cyclohexyl).

p-Acetylbenzenesulphonyl chloride was reacted with phosphorus pentachloride to yield *p*-(1-chlorovinyl)benzenesulphonyl chloride, smoothly converted to the sulphonamide (III; R=CCl:CH_2, R′=NH_2) with ammonia and thence to the sulphonylureas (I; R=CCl:CH_2, R′=Pr, Bu and cyclohexyl) by reaction with the appropriate isocyanates (Table 1).

Reaction of *p*-(2-chloroethyl)benzenesulphonamide (III; R=$CH_2{\cdot}CH_2{\cdot}$Cl, R′=NH_2) (Hayman, Petrow & Stephenson, 1964) with ethyl isocyanatoacetate yielded ethyl 5-[*p*-(2-chloroethyl)benzenesulphonyl] hydantoate (I; R=$CH_2{\cdot}CH_2{\cdot}$Cl, R′=$CH_2{\cdot}CO{\cdot}OEt$), which was converted by hot 6 N hydrochloric acid into the corresponding hydantoic acid (I; R=$CH_2{\cdot}CH_2{\cdot}$Cl, R′=$CH_2{\cdot}CO_2H$). Dehydrohalogenation of the latter compound with aqueous ethanolic sodium hydroxide gave 5-(*p*-vinylbenzenesulphonyl)hydantoic acid (I; R=CH:CH_2, R′=$CH_2{\cdot}CO_2H$). The ethyl ester of the latter compound was obtained directly by reaction of ethyl isocyanatoacetate with *p*-vinylbenzenesulphonamide.

Bromination of *p*-vinylbenzenesulphonamide (Hayman, Petrow & Stephenson, 1964) in acetic acid yielded the 1,2-dibromoethyl derivative (III; R=$CHBr{\cdot}CH_2Br$, R′=NH_2), which on treatment with hot ethanolic

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* The fifth paper in this series.

potash gave *p*-ethynylbenzenesulphonamide (III; R=C:CH, R′=NH_2), and this was purified *via* its silver salt. The compound had the same melting-point as *p*-acetylbenzenesulphonamide, but analysis, mixed melting-point and infra-red spectrum confirmed its identity. The sulphonylureas (I; R=C:CH, R′=Pr, Bu and cyclohexyl) were obtained by normal means (Table 1).

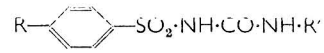
Stewart (1922) described *p*-sulphamoylcinnamic acid (III; R=CH:CH·CO_2H, R′=NH_2), obtained *via* chlorosulphonation of cinnamic acid, as a compound of m.p. 250–260°. Later, Burton & Hu (1949) obtained material of m.p. 276° by reaction of *p*-sulphamoylbenzaldehyde with malonic acid, whilst Müller (1949) obtained a compound of m.p. 285° by a Meerwein reaction between diazotised *p*-aminobenzenesulphonamide and acrylic acid. We have repeated Stewart's method of preparation, and obtained pure *p*-sulphamoylcinnamic acid, m.p. 275–277°, by alkaline hydrolysis of the readily purified methyl ester (III; R=CH:CH·CO·OMe, R′=NH_2). Preparation of the sulphonylbutylurea of the free acid (I; R=CH:CH·CO_2H, R′=Bu) was best accomplished by hydrolysis of the ethyl ester (I; R=CH:CH·CO·OEt, R′=Bu). The related diethylamide (I; R=CH:CH·CO·NEt_2, R′=Bu) was prepared by reaction of the di(acid chloride) (III; R=CH:CH·CO·Cl, R′=Cl) with diethylamine hydrochloride in refluxing chlorobenzene solution to yield the carboxyamide (III; R=CH:CH·CO·NEt_2, R′=Cl) (compare Jackman, Petrow, Stephenson & Wild, 1963). The latter compound was then converted *via* the sulphonamide (III; R=CH:CH·CO·NEt_2, R′=NH_2) into the sulphonylbutylurea by standard means (Table 1).

Acylated sulphonamides (III; R=Me or Cl, R′=NH·CO·Alk. or NH·CO·CH_2Ph) (Table 2) were obtained by refluxing the sulphonamide with the appropriate acid chloride in chlorobenzene solution.

Compounds bearing some formal resemblance to the active perhydro-azepinyl compound [I; R=Me, R′=$C_6H_{12}N$] were prepared by reaction of the sulphonylurethanes (II; R=Me or Cl, R′=OEt) with diamines of type NH_2·$[CH_2]_n$·R′ [where n=2, 3 or 4 and R′=NEt_2, perhydro-azepin-1-yl ($C_6H_{12}N$) or NH·Ph] (Table 3). The intermediate diamines were obtained by methods analogous to those described by Pozhil'tsova-Arbuzov (1953), Welvart (1955) and Mull & others (1958).

In connection with another research project, we had found that 4-chloro-3-nitrobenzenesulphonamide (IV; R=H) reacted with excess of hydrazine in boiling ethanol to yield 1-hydroxy-6-sulphamoyl-benztriazole (V; R=H) (cf. Müller; Müller & Zimmerman; Müller & Hoffmann, 1925). Reaction of this compound with 1 or 2 moles of butyl isocyanate in aqueous alkaline acetone yielded mixed products which contained none of the required sulphonylurea (V; R=CO·NH·Bu). The latter compound was, however, readily obtained by reaction of 1-butyl-3-(4-chloro-3-nitrobenzenesulphonyl)urea (IV; R=CO·NH·Bu) with excess of hydrazine in boiling ethanol. The corresponding carbonyl derivative (VI) was similarly obtained *via* 1-butyl-3-(4-chloro-3-nitrobenzoyl)urea. Reaction of the sulphonamide (IV; R=H) with phenylhydrazine gave the required sulphamoylbenztriazole (VII; R=H). The same compound was

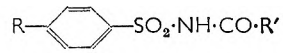
TABLE 1.



R	R′	m.p. (° C)	Formula	Found					Required				
				C	H	Cl	N	S	C	H	Cl	N	S
Me	$(C_3H_7)_2CH$	151–152	$C_{15}H_{24}N_2O_3S$	58·0	7·9		9·0	10·7	57·7	7·7		9·0	10·3
Me	$EtMe_2C$	128–130	$C_{13}H_{20}N_2O_3S$	55·1	7·0		9·9	11·4	54·9	7·1		9·9	11·3
Cl	$EtMe_2C$	140–142	$C_{12}H_{17}ClN_2O_3S$	47·5	5·3	12·0	9·0	10·5	47·3	5·6	11·6	9·2	10·5
Me	Et_3C	154–156	$C_{15}H_{24}N_2O_3S$	57·9	7·7		8·8	10·2	57·7	7·7		9·0	10·3
Me·CO	C_4H_9	156–157	$C_{13}H_{18}N_2O_4S$	52·5	6·1		9·4	10·6	52·3	6·1		9·4	10·7
$CH_2:C(Cl)$	C_3H_7	134–136	$C_{12}H_{15}ClN_2O_3S$	47·7	4·9	11·4	9·2	10·4	47·6	5·0	11·7	9·3	10·6
$CH_2:C(Cl)$	C_4H_9	136–138	$C_{13}H_{17}ClN_2O_3S$			11·0	8·7	10·3			11·2	8·8	10·1
$CH_2:C(Cl)$	Cyclohexyl	180–182	$C_{15}H_{18}ClN_2O_3S$	52·8	5·5	10·5	8·4	9·7	52·7	5·3	10·4	8·2	9·4
CH:C	C_3H_7	183–184	$C_{12}H_{14}N_2O_3S$	54·0	4·9		10·5	12·3	54·1	5·3		10·5	12·0
CH:C	C_4H_9	177–178	$C_{13}H_{16}N_2O_3S$				10·1	11·5				10·0	11·4
CH:C	Cyclohexyl	177–179	$C_{15}H_{18}N_2O_3S$	59·2	6·0		8·9	10·5	58·8	5·9		9·1	10·5
$HO_2C·CH:CH$	C_4H_9	217 (d.)	$C_{14}H_{18}N_2O_5S$	51·6	5·6		8·5		51·5	5·6		8·6	
$MeO·O·C·CH:CH$	Cyclohexyl	202–204	$C_{17}H_{22}N_2O_5S$	55·6	6·0		7·6	8·9	55·7	6·1		7·6	8·8
$EtO·O·C·CH:CH$	C_4H_9	129–131	$C_{16}H_{22}N_2O_5S$	54·5	6·4		8·2	9·0	54·2	6·3		7·9	9·1
$Et_2N·CO·CH:CH$	C_4H_9	188–190	$C_{18}H_{27}N_3O_4S$	56·8	7·0		10·8	8·4	56·7	7·1		11·0	8·4
H	4-Methylcyclohexyl*	178–180	$C_{14}H_{20}N_2O_3S$	57·0	6·7		9·6	10·8	56·7	6·8		9·5	10·8
Me	2-Methylcyclohexyl*	172–174	$C_{15}H_{22}N_2O_3S$	58·3	6·9		8·7	9·9	58·05	7·1		9·0	10·3
Cl	2-Methylcyclohexyl*	193–194	$C_{14}H_{19}ClN_2O_3S$	50·8	5·7	11·1	8·3	10·0	50·8	5·8	10·7	8·5	9·7

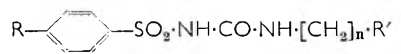
* *trans* isomer

TABLE 2.



R	R′	m.p. (° C)	Formula	Found					Required				
				C	H	Cl	N	S	C	H	Cl	N	S
Cl	Et	122–124	$C_9H_{10}ClNO_3S$	44·1	4·1	14·4	5·5	12·7	43·6	4·1	14·3	5·7	12·9
Cl	C_3H_7	99–101	$C_{10}H_{12}ClNO_3S$	46·0	4·3	13·6	5·5	12·2	45·9	4·6	13·6	5·4	12·3
Me	C_5H_{11}	79–81	$C_{13}H_{19}NO_3S$	58·1	6·9		5·3	11·7	57·9	7·1		5·2	11·9
Cl	C_5H_{11}	100–102	$C_{12}H_{16}ClNO_3S$	50·0	5·6		4·6		49·7	5·6		4·8	
Me	Et_2CH	96–98	$C_{13}H_{19}NO_3S$	58·4	7·0		4·9	12·0	57·9	7·1		5·2	11·9
Cl	Et_2CH	117–118	$C_{12}H_{16}ClNO_3S$	49·7	5·4	12·4	4·8	11·1	49·7	5·6	12·2	4·8	11·1
Me	$Ph{\cdot}CH_2$	149–151	$C_{15}H_{15}NO_3S$	62·0	5·3		5·0	11·4	62·3	5·2		4·8	11·1
Cl	$Ph{\cdot}CH_2$	152–153	$C_{14}H_{12}ClNO_3S$	54·5	4·0	11·9	4·7	10·7	54·3	3·9	11·4	4·5	10·4

TABLE 3.



R	R′	*n*	m.p. (° C)	Formula	Found					Required				
					C	H	Cl	N	S	C	H	Cl	N	S
Me	$C_5H_{10}N$	2	178–180	$C_{14}H_{20}N_2O_3S$	56·7	7·7		12·4	9·8	56·6	7·4		12·4	9·4
Cl	Et_2N	2	170–171	$C_{13}H_{19}ClN_2O_3S$	46·6	6·2	10·8	12·5	9·8	46·8	6·0	10·6	12·6	9·6
Cl	$C_5H_{12}N$	2	202–204	$C_{15}H_{20}ClN_2O_3S$	49·8	6·2	9·8	11·8	8·9	50·1	6·2	9·9	11·7	8·9
Cl	$C_5H_{12}N$	3	202–204	$C_{16}H_{24}ClN_2O_3S$	51·5	6·2	9·6	11·1	8·7	51·4	6·5	9·5	11·2	8·6
Cl	Ph·NH	3	144–145	$C_{16}H_{15}ClN_2O_3S$	52·7	5·2	9·8	11·3	8·6	52·2	4·9	9·6	11·4	8·7
Cl	$C_5H_{12}N$	4	184–185	$C_{17}H_{26}ClN_2O_3S$	52·1	6·5	9·2	11·0	8·2	52·6	6·8	9·1	10·8	8·3

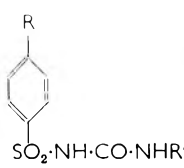
obtained from phenylhydrazine and the sulphonylurea (IV; R=CO·NH·Bu) due to fission of the urea group. Treatment of the sulphamoylbenztriazole (VII; R=H) with butyl isocyanate furnished the required butylsulphonylurea (VII; R=CO·NH·Bu). Both derivatives (VII; R=H or CO·NH·Bu) undoubtedly belong to the group of "meso-ionic" compounds (compare Goldstein & Voegeli, 1943 and Deorha & Joshi, 1961).

Further related benztriazoles, substituted in the 1-position by butyl, cyclohexyl or phenyl groups were obtained by reaction of the sulphonylurea (IV; R=CO·NH·Bu) with the appropriate amine to yield the nitro-amines (VIII; R=Bu, Ph or cyclohexyl, R′=NO_2). Reduction with sodium dithionite in aqueous ethanol gave the derived *o*-phenylenediamines (VIII; R=Bu, Ph or cyclohexyl, R′=NH_2), which were smoothly converted to the benztriazoles (IX; R=Bu, Ph or cyclohexyl) with nitrous acid. Additionally, 3-amino-4-butylaminobenzenesulphonamide was converted into the benzimidazole (X; R=H) by heating with formic acid, and this in turn yielded the required sulphonylurea (X; R=CO·NH·Bu).

p-Vinylbenzoic acid, prepared by an improved procedure (cf. Jäger & Waight, 1963), was converted *via* its acid chloride into 1-butyl-3-(*p*-vinylbenzoyl)urea (XII; X=O) by reaction with butylurea. The corresponding thiourea (XII; X=S) was also prepared.

Following the report on the hypoglycaemic activity of salicylic acid derivatives by Luthra & Tayal (1962) and the testing of benzimidazole derivatives for such activity (Tiwari & Swaroop, 1962) we prepared three imidazolines (XIII; R=H, OH or Me) compounds which contain features common to both salicylates and benzimidazoles.

Of the compounds described, only the sulphonylureas of type (I; R=C:CH or CCl:CH_2, R′=butyl or cyclohexyl) possessed significant hypoglycaemic activity (Dr. A. David, private communication).



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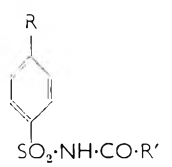

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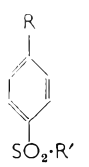

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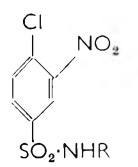

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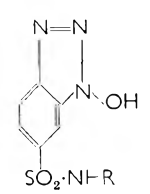
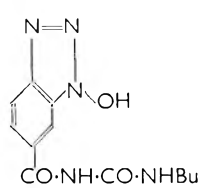


V



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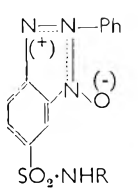

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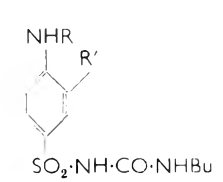

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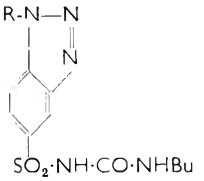
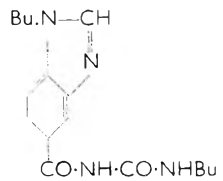


IX

X



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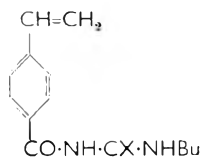

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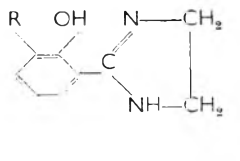

XIII

Experimental

Melting-points are uncorrected.

The first seven examples illustrate the methods used for the compounds listed in Tables 1 to 3, which also include analytical data.

1-(1-*Propylbutyl*)-3-*toluene-p-sulphonylurea.* A mixture of 4-aminoheptane (11·5 g) and methyl toluene-*p*-sulphonylcarbamate (11·4 g) was heated on the steam-bath for 8 hr. It was then cooled, diluted with water (500 ml) and acidified with concentrated hydrochloric acid (20 ml). The resultant viscous material was extracted with 5% sodium carbonate solution and filtered to remove unchanged sulphonamide. Acidification of the filtrate yielded the *product* (5·8 g), which had m.p. 151 to 152° after crystallisation from aqueous ethanol.

1,1-Di(2-*hydroxyethyl*)-3-*toluene*-p-*sulphonylurea.* A mixture of ethyl toluene-*p*-sulphonylcarbamate (18·2 g) and di(2-hydroxyethyl)amine (21 g) in chlorobenzene (100 ml) was heated at 110–120° for 4 to 5 hr. The mixture was then cooled, poured into water (200 ml) containing ammonia solution (50 ml, d=0·880), and extracted twice with ether. The aqueous layer was cooled and acidified with hydrochloric acid to yield the *product* (16·0 g), m.p. 107–108° (decomp.) [from acetone-light petroleum (b.p. 60–80°)]. Found: C, 47·3; H, 5·9; N, 9·3; S, 10·2. $C_{12}H_{18}N_2O_5S$ requires C, 47·7; H, 6·0; N, 9·3; S, 10·6%.

1-(p-*Chlorobenzenesulphonyl*)-3,3-di(2-*hydroxyethyl*)*urea* had m.p. 105–107° (decomp.) [from acetone-light petroleum (b.p. 60–80°)]. Found: C, 41·3; H, 4·6; Cl, 10·9; N, 8·8; S, 10·1. $C_{11}H_{15}ClN_2O_5S$ requires C, 40·9; H, 4·7; Cl, 11·0; N, 8·7; S, 9·9%.

1-*Butyl*-3-(p-*acetylbenzenesulphonyl*)*urea.* (*a*) A solution of *p*-aminoacetophenone (36·8 g) in concentrated hydrochloric acid (95 ml) and water (45 ml) was diazotised at 0° to 5° with a solution of sodium nitrite (20·7 g) in water (35 ml). The resultant solution was added to a stirred saturated

solution of sulphur dioxide in acetic acid (220 ml) containing cupric chloride dihydrate (11·8 g). When the vigorous reaction was complete the mixture was diluted with ice-water. The *product* was collected and washed with cold water. A portion, crystallised from toluene-light petroleum (b.p. 60–80°), had m.p. 86–88°. Found: C, 44·4; H, 3·1; Cl, 16·4; S, 14·9. p-*Acetylbenzenesulphonyl chloride*, $C_8H_7ClO_3S$ requires C, 43·9; H, 3·2; Cl, 16·2; S, 14·7%. (*b*) A solution of the foregoing sulphonyl chloride in chloroform (300 ml) was added with stirring to ammonia solution (300 ml, d=0·880) at room temperature to yield p-*acetylbenzenesulphonamide*, m.p. 180–181° (aqueous ethanol). Found: C, 48·3; H, 4·7; N, 7·0; S, 16·0. $C_8H_9NO_3S$ requires C, 48·2; H, 4·6; N, 7·0; S, 16·1%. (Yield 62% for the two stages). (*c*) To a solution of the sulphonamide (19·9 g) in acetone (250 ml) was added a solution of sodium hydroxide (4·0 g) in a minimum of water. The mixture was stirred, cooled to 0° and treated with butyl isocyanate (10·9 g) added during 10 min. Water (125 ml) was added after a short while to dissolve separated solids. After 1 hr the mixture was filtered, the filtrate diluted with ice-water (300 ml) and acidified to pH 6 with acetic acid. The *product* (77%) was collected, and washed with cold water. It had m.p. 156 to 157° after crystallisation from aqueous ethanol.

1-*Butyl*-3-[p-(1-*chlorovinyl*)*benzenesulphonyl*]*urea*. A solution of *p*-acetylbenzenesulphonyl chloride (40 g) in toluene (100 ml) was added with stirring during 20 min to a slurry of phosphorus pentachloride (50 g) in toluene (100 ml), and the mixture was heated on a steam-bath for 30 min. It was then cooled and added slowly with stirring to ammonia solution (800 ml, d=0·880). Stirring was continued for 30 min, after which time the solids (16·4 g) were collected and washed with water. The sulphonamide had m.p. 143–144° (decomp.) after crystallisation from aqueous ethanol. Found: C, 44·1; H, 3·4; Cl, 16·4; N, 6·6; S, 14·8. p-(1-*Chlorovinyl*)*benzenesulphonamide*, $C_8H_8ClNO_2S$, requires C, 44·1; H, 3·7; Cl, 16·3; N, 6·4; S, 14·7%. Reaction of the sulphonamide with butyl isocyanate in aqueous alkaline acetone, as described earlier, furnished the *product* (91% yield), m.p. 136–138° (from aqueous methanol).

Ethyl 5-[p-(2-*chloroethyl*)*benzenesulphonyl*]*hydantoate*. Ethyl isocyanatoacetate (27·1 g) was added during 15 min to a stirred mixture of *p*-(2-chloroethyl)benzenesulphonamide (44 g) in acetone (400 ml) and sodium hydroxide (8·0 g) in water (30 ml) at 0°–5°. When the addition was complete, stirring was continued for 4 hr at 25°, and then the mixture was poured into ice-cold water (3 litres) and filtered. Acidification of the filtrate with dilute hydrochloric acid yielded the *product* (63·2 g), m.p. 127–129° [from acetone-light petroleum (b.p. 60–80°)]. Found: C, 45·1; H, 5·0; Cl, 10·3; N, 7·9; S, 9·3. $C_{13}H_{17}ClN_2O_5S$ requires C, 44·75; H, 4·9; Cl, 10·2; N, 8·0; S, 9·2%.

5-[p-(2-*Chloroethyl*)*benzenesulphonyl*]*hydantoic acid*. The foregoing hydantoate (53·8 g) was heated on the steam-bath with 6 N hydrochloric acid (270 ml) for 1 hr. After cooling, the solids were collected, washed with water, and dissolved in 2% ammonia solution. The solution was filtered and the filtrate acidified with hydrochloric acid to yield the

product (45·4 g), m.p. 179–180° (decomp.) (from dilute ethanol). Found: C, 41·5; H, 4·0; Cl, 11·2; N, 9·0; S, 10·2. $C_{11}H_{13}ClN_2O_5S$ requires C, 41·2; H, 4·1; Cl, 11·1; N, 8·7; S, 10·0%.

5-(p-*Vinylbenzenesulphonyl*)*hydantoic acid.* A solution of the foregoing hydantoic acid (21·2 g) in ethanol (250 ml) was heated on the steam-bath and treated portionwise with a solution of sodium hydroxide (8·0 g) in water (30 ml). The reaction was completed by heating for 1 hr, and then the solvent was distilled off at reduced pressure. The residue was dissolved in hot water (200 ml) and filtered after the addition of charcoal. After cooling, the filtrate was acidified with dilute hydrochloric acid to yield the *product*, m.p. about 200° (decomp.) (from aqueous ethanol). Found: C, 46·7; H, 4·3; N, 9·5; S, 11·3. $C_{11}H_{12}N_2O_5S$ requires C, 46·5; H, 4·3; N, 9·9; S, 11·3%.

Ethyl 5-(p-*Vinylbenzenesulphonyl*)*hydantoate* was prepared by reaction of ethyl isocyanatoacetate (7·1 g) with *p*-vinylbenzenesulphonamide (9·15 g) in acetone (120 ml) containing sodium hydroxide (2·0 g) dissolved in water (5 ml). It had m.p. 173–4° (decomp.) (from ethanol). Found: C, 49·6; H, 5·2; N, 9·0; S, 10·2. $C_{13}H_{16}N_2O_5S$ requires C, 50·0; H, 5·2; N, 9·0; S, 10·3%.

1-*Cyclohexyl*-3-(p-*ethynylbenzenesulphonyl*)*urea.* (*a*) p-(1,2-*Dibromoethyl*)*benzenesulphonamide.* A solution of styrene-*p*-sulphonamide (89 g) in acetic acid (890 ml) was treated with stirring with a solution of bromine (80 g) in acetic acid (100 ml). The *product* (106 g), isolated by dilution with water (1,500 ml), had m.p. 177–179° (from methanol). Found: C, 27·7; H, 2·7; Br, 46·1; N, 3·9; S, 9·0. $C_8H_9Br_2NO_2S$ requires C, 28·0; H, 2·6; Br, 46·6; N, 4·1; S, 9·3%.

(*b*) p-*Ethynylbenzenesulphonamide.* A solution of the foregoing dibromo-derivative (21 g) in ethanol (250 ml) was treated with a solution of potassium hydroxide (16 g) in a minimum of water, and the mixture heated under reflux for 90 min. Water (250 ml) and acetic acid (25 ml) were then added, the excess of ethanol was boiled off and the mixture was filtered hot after the addition of charcoal. The solids (9·4 g) were dissolved in methanol (200 ml), stirred and treated with a solution of silver nitrate (10 g) in water (50 ml). The acetylide was collected, washed with methanol and added to a stirred solution of potassium cyanide (20 g) in water (200 ml). The pH of the mixture was adjusted to 9 with dilute nitric acid. The *product* (4·8 g) had m.p. 180–182° after crystallisation from water. Found: C, 52·7; H, 3·5; N, 8·0; S, 18·0. $C_8H_7NO_2S$ requires C, 53·0; H, 3·9; N, 7·7; S, 17·7%.

(*c*) 1-Cyclohexyl-3-(*p*-ethynylbenzenesulphonyl)urea was obtained by reaction of the foregoing sulphonamide with cyclohexyl isocyanate in aqueous alkaline acetone. It had m.p. 177–179° after crystallisation from aqueous ethanol.

Methyl p-*sulphamoylcinnamate* was obtained by esterification of crude *p*-sulphamoylcinnamic acid (Stewart, 1922). It had m.p. 188–190° (from aqueous methanol). Found: C, 49·6; H, 4·6; N, 5·4. $C_{10}H_{11}NO_4S$ requires C, 49·8; H, 4·6; N, 5·8%. Hydrolysis of the ester (4·84 g) with a solution of sodium hydroxide (4·8 g) in water (30 ml) on the steam bath

for 1 hr yielded, after acidification, pure *p*-sulphamoylcinnamic acid, m.p. 275–277° after crystallisation from acetic acid (compare Burton & Hu, 1949 and Müller, 1949)

Ethyl p-*sulphamoylcinnamate* had m.p. 133–135° (from aqueous ethanol). Found: C, 51·6; H, 5·1; N, 5·4; S, 12·4. $C_{11}H_{13}NO_4S$ requires, C, 51·8; H, 5·1; N, 5·5; S, 12·6%.

p-*Chlorosulphonylcinnamoyl chloride.* A solution of *p*-chlorosulphonylcinnamic acid (49·4 g) in 1,2-dichloroethane (300 ml) containing formdimethylamide (2 ml) was heated to boiling and treated with thionyl chloride (36 g), added during 30 min. The mixture was heated under reflux for 4 hr and then the volatile material was distilled off. The residual *product* had m.p. 116–118° after crystallisation from 1,2-dichloroethane—light petroleum (b.p. 60–80°). Found: C, 41·2; H, 2·4; Cl, 26·8; S, 11·7. $C_9H_6Cl_2O_3S$ requires C, 40·7; H, 2·3; Cl, 26·7; S, 12·1%.

p-Chlorosulphonylcinnamdiethylamide. A solution of the foregoing di(acid chloride) (5·32 g) in chlorobenzene (30 ml) was treated with diethylamine hydrochloride (2·2 g) and the mixture heated under reflux for 6 hr. Most of the solvent was distilled off at reduced pressure, and then dilution with light petroleum (b.p. 60–80°) furnished the *product* (5·5 g), m.p. 103–104°. Found: C, 51·6; H, 5·2; Cl, 12·0; N, 4·7; S, 10·6. $C_{13}H_{16}ClNO_3S$ requires C, 51·7; H, 5·3; Cl, 11·7; N, 4·6; S, 10·6%.

p-*Sulphamoylcinnamdiethylamide*, obtained in 88% yield by reaction of the foregoing sulphonyl chloride with liquid ammonia, had m.p. 193–195° (from aqueous methanol). Found: C, 55·6; H, 6·2; N, 10·2; S, 11·4. $C_{13}H_{18}N_2O_3S$ requires C, 55·3; H, 6·4; N, 9·9; S, 11·4%.

p-(*Butylcarbamoylsulphamoyl*)*cinnamdiethylamide*, obtained by reaction of the foregoing sulphonamide with butyl isocyanate in aqueous alkaline acetone, had m.p. 188–190° (from ethanol).

N-p-*Chlorobenzenesulphonylbutyramide.* A mixture of *p*-chlorobenzenesulphonamide (38·3 g), butyryl chloride (23·3 g) and chlorobenzene (100 ml) was heated under reflux for 1 hr. Dilution with light petroleum (b.p. 60–80°) furnished the *product* (45 g), m.p. 99–101° [from benzene—light petroleum (b.p. 60–80°)].

1-(p-*Chlorobenzenesulphonyl*)-3-(2-*perhydroazepin*-1′-*ylethyl*)*urea.* A solution of methyl *p*-chlorobenzenesulphonylcarbamate (12 g) in toluene (120 ml) was treated with 2-perhydroazepin-1′-ylethylamine (9 g) (Welvart, 1955), and the mixture heated at 100° for 3 hr. The toluene was distilled off at reduced pressure and the solid residue crystallised from water to yield the *product* (50%), m.p. 202–204°.

3-*Perhydroazepin*-1′-*ylbutyronitrile.* 3-Chlorobutyronitrile (52·0 g) was added during 30 min to a solution of hexamethyleneimine (99 g) in a mixture of benzene (100 ml) and chloroform (20 ml), and the mixture was heated under reflux for 3 hr. The mixture was cooled and water added to dissolve the imine hydrochloride. The organic layer was washed with water, and the solvent distilled off. The residual oil was distilled at 0·01 mm to yield the *product* (64 g), b.p. 80–82°; n_D^{24}, 1·4710. Found: C, 72·1; H, 10·7; N, 16·9. $C_{10}H_{18}N_2$ requires C, 72·2; H, 10·9; N, 16·9%.

1-(4-*Aminobutyl*)*perhydroazepine*. A solution of the foregoing nitrile (64 g) in ethanol (300 ml) was reduced at 100°–110° and 50 atmos. pressure with hydrogen, using Raney nickel as catalyst. The *product* (49 g) had b.p. 85–90° at 0·1 mm and n_D^{20}, 1,481. Found: C, 70·4; H, 12·9; N, 16·9. $C_{10}H_{22}N_2$ requires C, 70·5; H, 13·0; N, 16·5%.

1-*Butyl*-3-(4-*chloro*-3-*nitrobenzenesulphonyl*)*urea* was obtained in 98% yield by reaction of 4-chloro-3-nitrobenzenesulphonamide (47·4 g) in acetone (450 ml) containing sodium hydroxide (8 g) in water (20 ml) with butyl isocyanate (24 g), using the method described earlier. The *product* had m.p. 169–170° (from methanol). Found: C, 39·5; H, 4·1, N, 12·3. $C_{11}H_{14}ClN_3O_5S$ requires C, 39·4; H, 4·20; N, 12·5%.

1-*Butyl*-3-(1-*hydroxybenztriazole*-6-*sulphonyl*)*urea*. A solution of the foregoing sulphonylbutylurea (20·1 g) in ethanol (100 ml) was heated with hydrazine hydrate (18 g) under reflux for 3 hr with removal of ethanol over the last hour. The straw-coloured residue was dissolved in water (250 ml) and the solution acidified with hydrochloric acid. The *product* was collected and washed with water. It (16·2 g) had m.p. 169° (decomp.) after crystallisation from aqueous ethanol. Found: C, 41·8; H, 5·0; N, 22·2; S, 10·4. $C_{11}H_{15}N_5O_4S$ requires C, 42·2; H, 4·8; N, 22·4; S, 10·2%.

1-*Butyl*-3-(4-*chloro*-3-*nitrobenzoyl*)*urea*. Butylurea (28 g) was added to a solution of 4-chloro-3-nitrobenzoyl chloride (48 g) in toluene (400 ml) containing pyridine (5 drops), and the mixture was heated under reflux for 6 hr. The *product* (53·5 g) separated on cooling. It had m.p. 143–145° after crystallisation from ethanol. Found: C, 48·5; H, 4·9; Cl, 11·7; N, 13·9. $C_{12}H_{14}ClN_3O_4$ requires C, 48·1; H, 4·7; Cl, 11·8; N, 14·0%.

1-*Butyl*-3-(1-*hydroxybenztriazole*-6-*carbonyl*)*urea* was obtained in 65% yield when a solution of the foregoing compound (25 g) in ethanol (250 ml) was heated with hydrazine hydrate (25 g) under reflux for 8 hr. It had m.p. 220° (decomp.) (from aqueous ethanol).

4-*Butylamino*-3-*nitrobenzenesulphonamide*. A mixture of 4-chloro-3-nitrobenzenesulphonamide (23·7 g) and butylamine (25 ml) in ethanol (50 ml) was heated under reflux for 2·5 hr. The *product* (26·8 g) was collected and washed with cold ethanol. It had m.p. 184°–185° (from ethanol). Found: C, 43·8; H, 5·3; N, 15·4; S, 11·8. $C_{10}H_{15}N_3O_4S$ requires C, 43·9; H, 5·5; N, 15·4; S, 11·7%.

3-*Amino*-4-*butylaminobenzenesulphonamide*. A hot solution of the foregoing nitroamine (13·65 g) in water (130 ml) and ethanol (75 ml) was treated portionwise with a slurry of sodium dithionite (28 g) in water (40 ml) (compare Ashton & Suschitzky, 1957). The mixture was heated on the steam-bath for 1 hr and then diluted with water. The *product* (10·4 g), which crystallised, had m.p. 127–129° (from water). Found: C, 49·3; H, 7·1; N, 17·3; S, 13·2. $C_{10}H_{17}N_3O_2S$ requires C, 49·4; H, 7·0; N, 17·3; S, 13·2%.

1-*Butyl*-5-*sulphamoylbenztriazole*. A suspension of the foregoing diamine (8·4 g) in water (150 ml), ethanol (20 ml) and concentrated hydrochloric acid (4 ml) was stirred vigorously and treated during 15 min with a solution of sodium nitrite (3 g) in water (20 ml), and then the

reaction was completed by warming the mixture to 60° for 1 hr. The *product* (6 g) was collected and washed with water. It was crystallised from water after the addition of charcoal and had m.p. 134–136°. Found: C, 47·1; H, 5·5; N, 21·8; S, 12·6. $C_{10}H_{14}N_4O_2S$ requires C, 47·2; H, 5·5; N, 22·1; S, 12·6%.

1-*Butyl*-3-(1-*butylbenztriazole*-5-*sulphonyl*)*urea*, obtained in 90% yield by reaction of the foregoing compound with butyl isocyanate in aqueous alkaline acetone, had m.p. 140–141° (aqueous methanol). Found: C, 51·3; H, 6·9; N, 20·1; S, 8·9. $C_{15}H_{23}N_5O_3S$ requires C, 51·0; H, 6·6; N, 19·8; S, 9·1%.

1-*Butyl*-5-*sulphamoylbenzimidazole*. A mixture of 3-amino-4-butylaminobenzenesulphonamide (9·7 g) and formic acid (10 ml) was heated on the steam-bath for 3 hr, and then the excess of formic acid was distilled off at reduced pressure. Crystallisation of the residual solid from aqueous ethanol furnished the *product* (9·4 g), m.p. 160–162°. Found: C, 52·1; H, 5·8; N, 17·2. $C_{11}H_{15}N_3O_2S$ requires C, 52·2; H, 6·0; N, 16·6%.

1-*Butyl*-3-(1-*butylbenzimidazole*-5-*sulphonyl*)*urea* was obtained in 83% yield by reaction of the foregoing sulphonamide with butyl isocyanate under standard conditions. It had m.p. 189–190° (from ethanol). Found: N, 15·9; S, 8·8. $C_{16}H_{24}N_4O_3S$ requires N, 15·9; S, 9·1%.

1-*Butyl*-5-*sulphamoylbenzimidazol*-2-*one*. An intimate mixture of 3-amino-4-butylaminobenzenesulphonamide (5 g) and urea (5 g) was heated at 150° for 3 hr and the residue stirred with water (40 ml) during cooling. The *product* (4·8 g) had m.p. 199–201° (from ethanol). Found: C, 49·0; H, 5·5; N, 15·2. $C_{11}H_{15}N_3O_3S$ requires C, 49·1; H, 5·6; N, 15·6%.

4-*Cyclohexylamino*-3-*nitrobenzenesulphonamide*, obtained in 82% yield from 4-chloro-3-nitrobenzenesulphonamide, had m.p. 165–167° (from ethanol). Found: C, 48·5, H, 5·8; N, 14·2; S, 10·6. $C_{12}H_{17}N_3O_4S$ requires C, 48·2; H, 5·7; N, 14·0; S, 10·7%.

3-*Amino*-4-*cyclohexylaminobenzenesulphonamide* was obtained in 67% yield by reduction of the foregoing nitro-compound with sodium dithionite in aqueous ethanol. It had m.p. 138–139° (aqueous ethanol). Found: C, 53·5; H, 7·1; N, 15·6; S, 11·9. $C_{12}H_{19}N_3O_2S$ requires C, 53·2; H, 7·6; N, 15·9; S, 11·7%.

1-*Cyclohexyl*-5-*sulphamoylbenztriazole*, obtained in 96% yield by diazotisation of the foregoing amine, had m.p. 219–220° (from aqueous ethanol). Found: C, 51·2; H, 5·6; N, 20·1; S, 11·5. $C_{12}H_{16}N_4O_2S$ requires C, 51·4; H, 5·8; N, 20·0; S, 11·4%.

1-*Butyl*-3-(1-*cyclohexylbenztriazole*-5-*sulphonyl*)*urea* had m.p. 164–166° (aqueous ethanol). Found: C, 53·9; H, 6·6; N, 18·4; S, 8·4. $C_{17}H_{25}N_5O_3S$ requires C, 53·8; H, 6·6; N, 18·5; S, 8·5%.

2-*Nitro*-4-*sulphamoyldiphenylamine* (compare Fischer, 1891) had m.p. 181–183° (from aqueous methanol). Found: C, 49·6; H, 3·9; N, 14·4, S, 11·0. Calc. for $C_{12}H_{11}N_3O_4S$: C, 49·1; H, 3·8; N, 14·3; S, 10·9%.

2-*Amino*-4-*sulphamoyldiphenylamine* had m.p. 156–158° (aqueous methanol). Found: C, 54·6; H, 5·1; N, 16·3; S, 12·1. $C_{12}H_{13}N_3O_2S$ requires C, 54·7; H, 5·0; N, 16·0; S, 12·2%.

1-*Phenyl*-5-*sulphamoylbenztriazole* had m.p. 257–259° after precipitation from alkaline solution with hydrochloric acid. Found: C, 52·7; H, 3·5; N, 20·8. $C_{12}H_{10}N_4O_2S$ requires C, 52·5; H, 3·7; N, 20·4%.

1-*Butyl*-3-(1-*phenylbenztriazole*-5-*sulphonyl*)*urea*, m.p. 165–167° (from ethanol). Found: C, 54·3; H, 4·9; N, 19·2. $C_{13}H_{19}N_5O_3S$ requires C, 54·7; H, 5·1; N, 18·8%.

1-*Oxy*-2-*phenyl*-6-*sulphamoylbenztriazole* was obtained by heating a mixture of 4-chloro-3-nitrobenzenesulphonamide (23·7 g) and phenylhydrazine (32·4 g) in ethanol (100 ml) for 7 hr. It had m.p. 252° (decomp.) after washing with dilute hydrochloric acid and then with ethanol. Found: C, 49·7; H, 3·6; N, 18·9; S, 11·0. $C_{12}H_{10}N_4O_3S$ requires C, 49·7; H, 3·5; N, 19·3; S, 11·0%.

1-*Butyl*-3-(1-*oxy*-2-*phenylbenztriazole*-6-*sulphonyl*)*urea*, m.p. 182–184° (from ethanol). Found: C, 52·7; H, 4·7; N, 18·3; S, 7·9. $C_{17}H_{19}N_5O_4S$ requires C, 52·4; H, 4·9; N, 18·0; S, 8·2%.

p-*Vinylbenzoyl chloride*. A solution of *p*-vinylbenzoic acid (29·6 g) in dry benzene (70 ml) was treated with thionyl chloride (36 ml), the mixture heated under reflux for 1 hr, and then the excess of volatile material was distilled off on a steam-bath at *ca* 30 mm. The residual oil was treated with hydroquinone (0·5 g) and distilled at 0·5 mm to yield the *product* (21·4 g), b.p. 91–92°. Found: C, 65·0; H, 4·3; Cl, 20·7. C_9H_7ClO requires C, 64·9; H, 4·3; Cl, 21·3%.

1-*Butyl*-3-(p-*vinylbenzoyl*)*urea*. A solution of *p*-vinylbenzoyl chloride (8·2 g) in benzene (25 ml) was added during 10 min to a stirred solution of butylurea (6·4 g) and pyridine (4 ml) in benzene (75 ml) at 10°. Stirring was continued for 1 hr and the mixture was then heated to 60° for 1 hr further. It was then cooled, the precipitate of pyridine hydrochloride was filtered off and the filtrate was concentrated to about 25 ml at reduced pressure. The *product* (7 g) separated on dilution with light petroleum (b.p. 60–80°). It had m.p. 116–118° (from ethanol). Found: C, 68·2; H, 7·4; N, 11·3. $C_{14}H_{18}N_2O_2$ requires C, 68·3; H, 7·4; N, 11·4%.

1-*Butyl*-3-(p-*vinylbenzoyl*)*thiourea*. *p*-Vinylbenzoyl chloride (11·0 g) was added during 5 min to a stirred solution of ammonium thiocyanate (5·7 g) in acetone (40 ml) and the mixture heated under reflux for 5 min. It was then cooled and treated during 10 min with a solution of butylamine (4 g) in acetone (15 ml) and finally heated under reflux for 15 min. Most of the acetone was boiled off and the residue was then stirred with water (200 ml). The water was decanted off and the residual solid triturated with methanol to yield the *product* (3·7 g), m.p. 77–79° [from light petroleum (b.p. 60–80°)]. Found: C, 64·1; H, 7·2; N, 10·5; S, 12·2. $C_{14}H_{18}N_2OS$ requires C, 64·0; H, 6·9; N, 10·7; S, 12·2%.

2-(*o-Hydroxyphenyl*)*imidazoline*. Phenyl salicylate (42·8 g) was added carefully to 1,2-diaminoethane (13·2 g) and the mixture heated at 180° (internal temperature) for 1 hr. Phenol and some water were then distilled off under reduced pressure. The residue was dissolved in 2 N hydrochloric acid (400 ml) and filtered to remove about 3 g of 1,2-di(*o*-hydroxybenzamido)ethane. The filtrate was evaporated to dryness at reduced pressure, and the residue dissolved in water and neutralised with

ammonia solution to yield the *product* (21·8 g), m.p. 207–209° (from methanol). Found: C, 66·5; H, 6·2; N, 17·5. Calc. for $C_9H_{10}N_2O$, C, 66·6; H, 6·2; N, 17·3%.

2-(2,3-*Dihydroxyphenyl*)*imidazoline hydrochloride* was obtained in 53% yield by reaction of methyl catechuate with 1,2-diaminoethane at 160–170° for 1 hr at about 100 mm pressure. It had m.p. 272–274° (decomp.) (from ethanol-ether). Found: C, 50·5; H, 5·2; Cl, 16·1; N, 13·0. $C_9H_{11}ClN_2O_2$ requires C, 50·4; H, 5·2; Cl, 16·5; N, 13·05%.

2-(2-*Hydroxy*-m-*toiyl*)*imidazoline*, obtained in 52% yield by reaction of phenyl 2-hydroxy-*m*-toluate with 1,2-diaminoethane, had m.p. 260–262° (from 2-ethoxyethanol). Found: C, 68·0; H, 6·6; N, 16·2. $C_{10}H_{12}N_2O$ requires C, 68·2; H, 6·9; N, 15·9%.

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Effect of an anti-acetylcholine drug, methscopolamine bromide, on ulcer formation and gastric mucus

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A 30-hr period of restraint in rats was followed by the appearance of ulcers in the gastric corpus coincidental with a marked reduction of gastric juice volume, acid and also of hexosamine which was used as an estimation of mucus content. Methscopolamine (Pamine), an anti-acetylcholine drug, prevented ulcer formation, reduced further volume and acid output but produced a 3-4 fold increase in hexosamine concentration. Tissue (corpus and antrum) hexosamine was moderately reduced by restraint. In the corpus, this was counteracted by methscopolamine but antrum hexosamine was not influenced by this drug. The anti-ulcer property of methscopolamine may be due not only to its effect on acid secretion but also to the rise in gastric mucus concentration that it produced.

ATROPINE-LIKE drugs are widely used for peptic ulcer therapy on the assumption that the reduction in acid secretion produced by these substances favours the healing of ulcers. Indeed, these compounds have been found to protect animals against a variety of experimentally-induced ulcers, such as those produced by fasting (Visscher, Seay, Tazelaar, Veldkamp & Vander Brook, 1954), pylorus ligation (Lehman & Stefko, 1949; Kowalewski, Mackenzie, Shnitka & Bain, 1954; Visscher and others, 1954), corticosteroids (Robert & Nezamis, 1959), histamine (Lehman & Stefko, 1949), reserpine (Blackman, Campion & Fastier, 1959; LaBarre, 1959), 5-hydroxytryptophan (Haverback & Bogdanski, 1957) and restraint (Hanson & Brodie, 1960). It must be noted that in these experiments, anti-acetylcholine drugs were given to prevent, not to cure, ulcers. There seems to be little doubt that the presence of acid in the gastric juice can hinder the healing of an ulcer. Whether hyperacidity, however, initiates peptic ulcer is debated and appears doubtful. Other factors, perhaps equally important, may be considered to increase or decrease the resistance of gastric and duodenal mucosa without much change in acid secretion. Among these, the amount and rate of secretion of gastroduodenal mucus has been implicated in the past in the natural defence against ulcerogenic agents (Hollander, 1951; Robert & Nezamis, 1959, 1963, 1964; Robert, Bayer & Nezamis, 1963).

It was thought that besides their antisecretory property, atropine-like agents might protect the stomach by increasing the mucus content of gastric juice. Actually, it was noted in an earlier study that the gastric mucosa of fasted rats treated with methscopolamine bromide (Pamine) "was covered with a thicker layer of mucus than that of control animals" (Robert & Nezamis, 1959).

We have now investigated the influence of methscopolamine bromide on the mucus content of gastric juice and tissue and on gastric juice acidity of restrained and nonrestrained rats. Restraint was previously shown to produce ulcers in rats (Rossi, Bonfils, Lieffogh & Lambling, 1956; Brodie & Hanson, 1960). A correlation was attempted of the anti-ulcer effect observed and the changes in gastric juice and tissue composition.

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Method

Separate experiments were made for gastric juice and for gastric tissue analysis. In each, 15 female Sprague-Dawley rats, weighing 190 to 205 g, were used per group.

GASTRIC JUICE

Animals were fasted at 4.00 p.m. They were first placed in individual cages for 24 hr, then transferred into stainless steel tubes during the night preceding pylorus ligation (Robert & others, 1963). After this day and a half of fasting, the pylorus was ligated under ether anesthesia, the abdominal incision closed with surgical clips and the rats returned to the metal tubes for a period of 5 hr, after which they were killed with chloroform. This procedure avoided the contamination of gastric juice by eating faeces or hair.

Other animals were fasted for the same duration but after the first night of fasting, they were immobilized on their back on linoleum boards with adhesive tape. 24 hr later, the pylorus was ligated under ether anaesthesia while the animals were still immobilised and they were killed 5 hr later with chloroform.

Methscopolamine bromide was administered subcutaneously at the dose of 0·5 mg/0·2 ml (2·5 mg/kg) per injection, in saline solution. A total of three injections was given: at the time of restraint, 8 hr, and 24 hr later, with the last injection made immediately before pylorus ligation. The controls, either fasted only or restrained, received saline injections. At autopsy the oesophagus was clamped, the stomach washed with deionised water, dried with a towel and the contents emptied into a graduated cylinder through an incision of the forestomach. In methscopolamine-treated animals, there was not enough gastric juice to permit chemical analysis; therefore, 2 ml of deionised water was first injected, from a calibrated syringe, through the cardia into the stomach before dissecting it. The juice of these animals was further diluted with water up to 3 ml. The stomachs were later opened along the greater curvature and examined with a 2× magnifier for the presence of ulcerations.

GASTRIC TISSUE

The conditions of the animals were identical to those for gastric juice studies except that the pylorus was not ligated. Duration of the experiment, restraint and treatment with methscopolamine, were the same. At termination, the stomach was dissected, opened along the greater curvature and washed with lukewarm running tap water and then examined for the presence of ulcerations. The fore-stomach was separated and discarded and the antrum was cut out. Both corpus and antrum were dried separately to constant weight (overnight at 75°).

CHEMICAL ANALYSIS

Hexosamine in gastric juice and tissue was determined and used as an estimation of mucus content. A modification (Robert & others, 1963)

of Boas method (Boas, 1953) was used. Results are expressed, for the juice, in mg/ml (concentration) and mg/5 hr (output) and for the tissues in mg/100 mg of dry weight. Free and total acid output was measured using Topfer's reagent and phenolphthalein and expressed in m-equiv./5 hr.

Results

GASTRIC JUICE (Table 1)

Restraint alone reduced the volume of secretion by 34%. Administration of methscopolamine to either restrained or unrestrained rats produced a reduction of 90% from the level of saline injected animals. The output/5 hr of free and total acid was decreased by about 50% after restraint and it was almost completely inhibited (83–93%) by methscopolamine in both restrained and unrestrained animals.

Hexosamine concentration was not changed by restraint but was greatly increased (3–4 fold) by methscopolamine. Total hexosamine output, on the other hand, was reduced by 38% in restrained animals. Methscopolamine produced a 51–56% reduction in output in all rats.

TABLE 1. GASTRIC JUICE HEXOSAMINE AND ACIDITY AFTER RESTRAINT. EFFECT OF METHSCOPOLAMINE

	Unrestrained		Restrained			
	I Saline	II Methscopol-amine	III Saline	IV Methscopol-amine	% Change	P*
Exp. 1						
No. of animals	14	15	13	15		
Initial weight g	199	198	198	198		
Final weight g	165	165	164	167		
Gastric juice Volume (ml)	6·7 ± 0·3	0·7 ± 0·04	4·4 ± 0·5	0·4 ± 0·03	I–II −90 III–IV −91 I–III −34	<0·01 <0·01 <0·01
Hexosamine mg/ml	0·318 ± 0·02	1·416 ± 0·07	0·316 ± 0·02	1·583 ± 0·09	I–II +345 III–IV +402 I–III −1	<0·01 <0·01 >0·05
mg/5 hr	2·110 ± 0·1	0·933 ± 0·03	1·303 ± 0·1	0·635 ± 0·04	I–II −56 III–IV −51 I–III −38	<0·01 <0·01 <0·01
Acid output (m-equiv./5 hr) Free acid	0·681 ± 0·03	0·051 ± 0·003	0·339 ± 0·04	0·031 ± 0·002	I–II −93 III–IV −91 I–III −50	<0·01 <0·01 <0·01
Total acid	0·881 ± 0·04	0·119 ± 0·006	0·458 ± 0·05	0·077 ± 0·006	I–II −87 III–IV −83 I–III −48	<0·01 <0·01 <0·01
Ulcer incidence %	0	0	64	0		

* P value, obtained from a "*t*" test comparing the means of two groups.

GASTRIC TISSUE (Table 2)

Hexosamine concentration in restrained animals was slightly decreased in the corpus (7%); the reduction was more marked in the antrum (16%).

Both changes were statistically significant. Methscopolamine exerted the opposite effect in the corpus (increase of 14%), whereas it did not affect hexosamine content of the antrum.

TABLE 2. GASTRIC TISSUE HEXOSAMINE AFTER RESTRAINT. EFFECT OF METHSCOPOLAMINE

	Unrestrained		Restrained			
	I Saline	II Methscopolamine	III Saline	IV Methscopolamine	% Change	P*
Exp. 2						
No. of animals	15	15	15	15		
Initial weight g	195	195	195	196		
Final weight g	166	168	167	168		
Hexosamine mg/100 mg dry tissue						
Corpus	0·988 ± 0·02	1·129 ± 0·02	0·915 ± 0·02	1·044 ± 0·02	I–II +14 III–IV +14 I–III −7	<0·01 <0·01 <0·05
Antrum	2·202 ± 0·05	2·052 ± 0·06	1·849 ± 0·06	1·884 ± 0·06	I–II −7 III–IV +2 I–III −16	>0·05 >0·05 <0·01
Ulcer incidence	0	0	74	14		

* P value, obtained from a "t" test comparing the means of two groups.

ULCER FORMATION (Tables 1 and 2)

In both experiments the incidence of ulcers was about the same in restrained, untreated animals. It is noteworthy that the ulcers appeared only in the corpus, the antrum always being intact. Methscopolamine completely prevented ulcer formation in the first experiment; in the second, only 3 out of 15 rats had ulcers (20%) and these were single and very small. This confirms results obtained by Hanson & Brodie (1960) using other anti-acetylcholine agents.

Discussion

Inhibition by restraint of the volume and acidity of gastric juice observed in these experiments agrees with results obtained by Brodie, Marshall & Moreno (1962), although the change in acidity was greater in our experiments. It was not as marked, however, as that reported by Menguy (1960) (94%). Such minor discrepancies in these three studies can be ascribed to differences in techniques and include factors like sex, body weight, restraint procedure, interval between times of pylorus ligation and killing.

The most interesting finding was the marked reduction in hexosamine output in gastric juice (first experiment) in animals developing restraint ulcers. Hexosamine was used as an index of mucus content. There is no good method for measuring the amount of mucus, but hexosamine, although present in most mucoproteins, was found to relate very well with histological localisation of mucus in many organs (Robert & others, 1963). The results of the second experiment indicate that after a 30-hr

period of restraint, the mucosa itself synthesises less mucus than it normally does since concentration of hexosamine in the corpus and especially in the antrum was reduced. It is probable that the real diminution in corpus mucus was greater than that actually measured because this portion of the stomach contains abundant parietal and chief cells which are not expected to contain appreciable amounts of hexosamine. The antrum mucosa, on the other hand, is composed entirely of mucus glands. This conclusion is supported by determination of hexosamine in superficial scrapings of corpus mucosa, previously reported (Robert & others, 1963). Scrapings, composed almost exclusively of mucus cells, were found to contain 2·5 times more hexosamine than the whole wall of the corpus.

Although methscopolamine depressed secretory activity of the stomach measured by volume, acid and mucus output, it increased the concentration of hexosamine in the juice over 3 fold in unrestrained and 4 fold in restrained rats. This suggests that the ulcer-preventing property of this compound could be due not only to its acid-suppressing effect but also to its action on mucus. Methscopolamine is not a mucigogue, since it reduced the total output of mucus, but whatever juice was still being secreted was viscous and very rich in mucus. It is possible that such a thick secretion, spread over the mucosa, constituted a mucus barrier that contributed in preventing the development of ulcers. Piper, Stiel & Fenton (1962) observed a similar rise in mucus concentration in gastric juice of psychiatric patients given anti-acetylcholine drugs and undergoing insulin hypoglycaemia therapy. They found that the increase in mucus coincided with a fall in volume and acidity.

Finally, the fact that the antrum was not ulcerated following restraint is worth mentioning. The mucus (hexosamine) concentration of this portion is twice that of the corpus (Table 2). It is suggested that the two findings are related. Even though antrum hexosamine of restrained animals was reduced by 16%, so much remained in the mucosa that it may have been enough to protect it from ulcerating. A similar correlation was observed also in the case of ulcers due to prolonged fasting (Robert & others, 1963).

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Spectrophotometric determination of isoniazid in excess *p*-aminosalicylate

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The presence of a large excess of *p*-aminosalicylic acid prevents the direct application of Vierordt's spectrophotometric method to the determination of isoniazid in tablets. The method can, however, be applied after the bulk of the *p*-aminosalicylic acid has been removed with ether. The extinction of the aqueous phase containing all the isoniazid and some of the *p*-aminosalicylic acid is then determined at two wavelengths and the concentration of isoniazid calculated therefrom.

THE determination of isoniazid in mixtures with sodium *p*-aminosalicylate has received much attention and methods involving precipitation, gasometry, non-aqueous titrations, polarographic and colorimetric procedures have been critically reviewed by Mitchell, Haugas & McRoe (1957). Lee & Ho (1962) found the method of Mitchell & others (1957), which involved the reduction of isoniazid with a zinc-copper couple, to give erratic results when applied to tablets. In their modification of this method, Lee & Ho removed interfering tablet material with hydrochloric acid and obtained better results for tablets. However, this modified method is more involved and the authors have not reported the recovery of small amounts of isoniazid in *p*-aminosalicylate (about 5 mg of isoniazid in 0·5 g of *p*-aminosalicylate which was found by Mitchell & others to give smaller recoveries due to errors in small titre differences).

In a search for a method which did not involve reduction, distillation, colorimetry, extraction and weighing, the application of the spectrophotometric analysis to a two-component mixture was considered. At the onset it was evident that because of the large excess of *p*-aminosalicylate associated with isoniazid in commercial samples a direct spectrophotometric method would be of no avail. Investigations with extraction procedures revealed that with mixtures of *p*-aminosalicylate-isoniazid about 50:1, the bulk of *p*-aminosalicylate could be removed by ether from an acid solution of the mixture. The aqueous phase then contained some *p*-aminosalicylate and all of the isoniazid. The application of the spectrophotometric analysis for the estimation of isoniazid in the presence of small quantities of *p*-aminosalicylate in this aqueous phase was found to give satisfactory results.

Glenn (1960) derived equations for the concentration of two components in a mixture, viz

$$C_A = E_1/\alpha_1 \left[\frac{b - m}{b - a}\right] \quad .. \quad .. \quad .. \quad (1)$$

$$C_B = E_2/\beta_2 \left[\frac{b\,(m - a)}{m\,(b - a)}\right] \quad .. \quad .. \quad (2)$$

where the subscripts "1" and "2" refer to wavelengths, E denotes extinction of a 1 cm layer of the solution of a mixture of A and B, C_A and

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C_B are concentrations of A and B, α and β are their respective extinction coefficients, and

$$m = E_2/E_1,\ a = \alpha_2/\alpha_1,\ b = \beta_2/\beta_1$$

where m refers to the mixture, a to substance A and b to substance B.

The condition for the application of the above equations is that both A and B must be absorbing substances which do not interact with each other, and the ratio b/m should be outside the limit 0·1–2·0. These conditions were found to be fulfilled for the acid solution after extraction with ether. Swietoslawska (1956) has derived expressions for calculating the analytical error for variable a, b and concentration of C_A and C_B. For maximum accuracy of C_A and C_B, Berry (1945) has shown that α_1/β_1 should be a maximum and α_2/β_2 should be a minimum. By drawing a graph of α/β against wavelength it is possible to choose a maximum and minimum of the plot as the two best wavelengths 1 and 2. For isoniazid and *p*-aminosalicylate, these wavelengths were found to be 255 mμ ("1") and 305 mμ ("2"). For the present purpose "A" was chosen to refer to isoniazid and "B" to *p*-aminosalicylate.

The extinction coefficients, E (1%, 1 cm) of isoniazid (α_1,α_2) and *p*-aminosalicylate (β_1,β_2) in 0·01 N hydrochloric acid were determined at 255 and 305 mμ from which the a and b values calculated were used for all the determinations. However, it must be pointed out that the a and b values can simply and rapidly be determined from solutions of "A" and "B" of unknown concentration. For the first assay using the proposed method it would, of course, be more convenient to calculate the a and b values from extinctions of the standard solutions. C_A and C_B would be in g/100 ml solution.

Specific example: Commercial sample claiming 15 mg of isoniazid and 0·5 g of sodium *p*-aminosalicylate per tablet. Average weight of one tablet, 0·5995 g. Weight of powdered tablet taken for analysis, 0·1982 g. Acid solution after extraction with ether, diluted to 400 ml.

$$E_1 = 0{\cdot}477;\quad E_2 = 0{\cdot}268;$$
$$m = E_2/E_1 = 0{\cdot}5618;$$
$$\alpha_1 = 329;\ \alpha_2 = 64{\cdot}5;\quad a = \alpha_2/\alpha_1 = 0{\cdot}1960;$$
$$\beta_1 = 112;\ \beta_2 = 378;\quad b = \beta_2/\beta_1 = 3{\cdot}375;$$
$$b/m = 6{\cdot}0$$

$$C_A = E_1/\alpha_1 \left[\frac{b - m}{b - a}\right] = \frac{0{\cdot}477}{329}\left[\frac{3{\cdot}375-0{\cdot}5618}{3{\cdot}375-0{\cdot}1960}\right]$$
$$= 0{\cdot}001283 \text{ g/100 ml}$$

i.e. concentration of isoniazid is 0·001283 g/100 ml of solution. Therefore,

$$\text{isoniazid content per tablet} = 0{\cdot}001283 \times 4 \times 1000 \times \frac{0{\cdot}5995}{0{\cdot}1982}$$
$$= 15{\cdot}52 \text{ mg.}$$

$$C_B = E_2/\beta_2 \left[\frac{b\,(m - a)}{m\,(b - a)}\right] = \frac{0{\cdot}268}{378}\left[\frac{3{\cdot}375\,(0{\cdot}5618 - 0{\cdot}1960)}{0{\cdot}5618\,(3{\cdot}373 - 0{\cdot}1960)}\right]$$
$$= 0{\cdot}0004901 \text{ g/100 ml}$$

i.e. concentration of *p*-aminosalicylate is 0·0004901 g/100 ml of solution. Therefore *p*-aminosalicylate content per tablet (in aqueous solution)

$$= 0{\cdot}0004901 \times 1000 \times 4 \times \frac{0{\cdot}5995}{0{\cdot}1982} = 5{\cdot}930 \text{ mg}$$

This calculation is intended merely to show the small quantity of *p*-aminosalicylic acid left in the aqueous phase, indicating the efficiency of the ether extraction process.

Experimental

Extinction coefficients. 0·001% solutions of isoniazid and *p*-aminosalicylate in 0·01N hydrochloric acid are prepared and their extinctions measured at 255 and 305 mμ. These extinctions multiplied by 1000 gives the extinction coefficients E (1%, 1 cm) at the two wavelengths of each of the two substances. These extinction coefficients together with the ratios a and b should be revised from time to time. Since the ratios a and b do not depend on the concentrations, their revision merely requires preparation of solutions of unknown concentration and measuring the extinctions at 255 mμ and 305 mμ with the proviso that the extinctions be within a reasonable range (e.g. 0·2–1·0).

Procedure. Weigh and powder 20 tablets. Accurately weigh an aliquot of powder containing about 4 mg of isoniazid, transfer to a separating funnel. Add 0·1N hydrochloric acid (20 ml) and shake the funnel for about 2 min. The mixture is then extracted with 4 × 30 ml ether. The combined ether extracts are washed with water (5 ml) and the washings transferred to the aqueous phase. The combined aqueous solutions are diluted to 200 ml. Dilute 25 ml of this solution to 50 ml with 0·01N hydrochloric acid. Determine the extinction of this solution as soon as possible at 255 and 305 mμ (*p*-aminosalicylic acid in solution undergoes decomposition on prolonged standing). The concentrations of isoniazid and *p*-aminosalicylate in the aqueous phase are then calculated from equations (1) and (2).

Results

TABLE 1. ESTIMATION OF ISONIAZID IN THE PRESENCE OF 0·1 g AMOUNTS OF SODIUM *p*-AMINOSALICYLATE

Weight of isoniazid taken (mg)	Isoniazid recovered	
	mg	%
1	1·00	100·0
2	1·97	98·5
3	2·97	99·0
4	3·96	99·0
5	4·95	99·0
6	5·93	98·9
7	6·97	99·6
8	8·07	100·9

The method was applied to synthetic mixtures of varying quantities of isoniazid in the presence of sodium *p*-aminosalicylate and the results obtained are given in Table 1.

TABLE 2. ESTIMATION OF ISONIAZID IN TABLETS

Weight per tablet of sodium *p*-aminosalicylate (g) as stated	Weight per tablet of isoniazid (mg)		
	Stated	Found	
		(mg)	%
0·5	15	15·6	104
0·5	15	15·5	103
0·5	15	15·6	104
0·5	15	15·5	103
0·5	15	15·6	104

Five commercial samples in tablet form were analysed by the proposed method and the results are given in Table 2.

Discussion

Recoveries of isoniazid from tablets obtained by the proposed method (Table 2) are higher than those obtained by Lee & Ho (1962). Recoveries of isoniazid from synthetic mixtures (Table 1) containing as much as 100 times more *p*-aminosalicylate are excellent. The proposed method is therefore not sensitive to error for small amounts of isoniazid with *p*-aminosalicylate as in the method of Mitchell & others (1957).

Using equation (2), the quantity of *p*-aminosalicylate remaining in the aqueous phase was found to vary between 1 and 6 mg both for synthetic mixtures and tablets.

Acknowledgement. We wish to thank Mr. Chia Chwee Leong, Chief Chemist, Singapore, for his interest.

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Letters to the Editor

Effect of aryl isothiocyanates on ^{131}I uptake by the mouse thyroid gland*

SIR,—Thiocyanate salts are known to block iodine uptake by the thyroid gland, and we wondered if aryl isothiocyanates might also exert this effect.

Groups of seven male mice (Swiss-Webster type) weighing about 28 g were treated orally with 150 mg/kg of α-naphthyl, β-naphthyl, or phenylisothiocyanates. According to VanderLaan & VanderLaan (1947), the animals were then immediately injected with propylthiouracil, 2 mg/mouse, to prevent the binding of ^{131}I by thyroglobulin. 30 min later the animals were treated with 2 μc ^{131}I per mouse. The animals were killed 90 min later, at 2 hr after treatment with the aryl isothiocyanates. Whole blood was collected and heparinised. Rather than attempt to isolate individual mouse thyroid glands, a section of trachea with the adhering glands was taken.

The radioactivity of the trachea block and of blood was measured in a Baird-Atomic scintillation detector, Model 810B and Baird-Atomic scaler, Model 132. Counts per μl of blood were converted to counts per μl of serum by use of a factor (1·33) to account for the small uptake of ^{131}I by erythrocytes. The thyroid (iodide) space was then calculated.†

Statistical analysis was by an analysis of variance and the Tukey test for significance of the differences of the means (Snedecor, 1956). Results are shown in Table 1.

TABLE 1. EFFECT OF ARYL ISOTHIOCYANATES ON THYROID UPTAKE OF ^{131}I

Treatment	Thyroid space (μl)*
α-Naphthylisothiocyanate	421 ± 51†
Phenylisothiocyanate	321 ± 26
Untreated control	288 ± 40
β-Naphthylisothiocyanate	254 ± 24

* Mean ± standard error.
† Mean value is significantly larger than that of the untreated control group at the P0·05 level.

Phenylisothiocyanate and β-naphthylisothiocyanate did not significantly alter thyroid space. However, the results indicated that α-naphthylisothiocyanate enhanced, rather than blocked, iodide uptake by the thyroid. Hence, α-naphthylisothiocyanate has, apparently, altered the blood-thyroid barrier in respect to iodide transport into the thyroid gland in some unexplained fashion. The action of α-naphthylisothiocyanate on the blood-thyroid barrier is rapid since the effect was demonstrable 2 hr after oral administration of the drug. Similar rapidity of α-naphthylisothiocyanate action has also been observed in respect to onset of hyperbilirubinaemia in mice (Becker & Plaa, 1963). Because of this rapid action, experiments on the effects of α-naphthylisothiocyanate on

* Supported in part by United States Public Health Service grant No. AM-8083 and Special Fellowship AM-19, 889 from the National Institute of Arthritis and Metabolic Diseases.

† Thyroid iodide space (μl) = $\frac{[\text{thyroid block (cpm)}—\text{background (cpm)}]\ 100}{1{\cdot}33\ [\text{blood (cpm/100 μl)}—\text{background (cpm)}]}$

other physiological barriers seems warranted, as are investigations of the action of α-naphthylisothiocyanate and related aryl isothiocyanates and thiocyanates on the thyroid gland.

Thanks are due to Drs. N. S. Halmi and J. Scranton for the use of equipment and the radioiodide employed.

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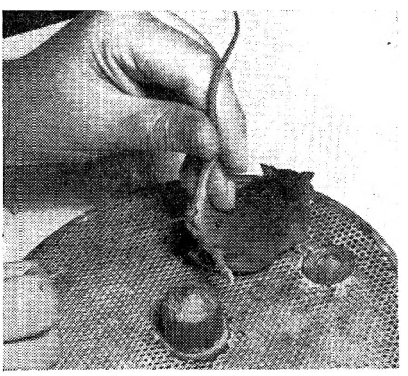
Measuring the temperature of a mouse

SIR,—It was pointed out by McLaren in 1961 that the body temperatures of mice could be measured successfully in the rectum, if the mouse were placed on a wire grid and held by the tail. This method, when carried out correctly, causes the least possible restraint of the animal as is clear from Fig. 1. It is



difficult to envisage that holding an animal to measure its body temperature by means of an oesophageal thermocouple (Brittain & Spencer 1964) could cause less restraint.

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Influence of drugs on $^{47}Ca^{2+}$ release from depolarized intestinal smooth muscle

SIR,—Schatzmann (1963) noted that acetylcholine or excess potassium increased the rate of $^{45}Ca^{2+}$ exchange in the taenia coli. Durbin & Jenkinson (1961a) have shown that carbachol-stimulation of the depolarized taenia coli does not alter $^{45}Ca^{2+}$ efflux, and more recently von Hattingberg & Rahn (1964) have found that $^{47}Ca^{2+}$ release from the taenia coli was increased by papaverine.

Using strips of longitudinal muscle from the guinea-pig ileum, depolarized in potassium-rich Krebs solution, we have observed the effects upon $^{47}Ca^{2+}$ release of a group of drugs which act upon smooth muscle. The muscle was prepared according to Ambache (1954) and Weiss, Coalson & Hurwitz (1961). Four 2 cm long pieces from the same length of muscle were used for each series. Adjacent pieces served as test and control. Each strip was stretched by a 0·2 g weight, incubated for 30 min at 37° in Krebs solution and then for not less than 2 hr in radioactive, potassium-rich Krebs solution at the same temperature. After washing (30 sec) in a stream of non-radioactive, potassium-rich Krebs solution, each pair of strips was exposed for 8 consecutive 1 min periods to tubes containing 5 ml of non-radioactive potassium-rich Krebs solution: the 4th and 7th tubes of one series contained the drug, those of the parallel series, the control solution. With papaverine, (Table 1) the exposure period was lengthened to 10 min to allow relaxation to be more complete; with adrenaline (Table 1), this was to attempt to obtain a significant relaxation. Each solution was counted (100 sec; thallium-activated sodium iodide crystal scintillation counter; ECKO type N597) and the total counts released added to those remaining in the tissue at the end of the experiment. The % counts released during exposure to the drug were calculated and compared with the control. The difference between control and drug-treated muscles was tested for significance using Student's "*t*" test. The results are shown in Table 1. These confirm the findings of Durbin & Jenkinson (1961a) with respect to carbachol. Acetylcholine and histamine behaved similarly to carbachol. Adrenaline

TABLE 1. RELEASE OF $^{47}Ca^{2+}$ BY DEPOLARIZED STRIPS OF THE LONGITUDINAL MUSCLE OF THE GUINEA-PIG ILEUM

Drug	Dose (μg/ml)	No. of pairs	Effect on $^{47}Ca^{2+}$ release		Mechanical response
			4th min	7th min	
Acetylcholine chloride	10	24	No change $0{\cdot}30 < P < 0{\cdot}40$	No change $0{\cdot}70 < P < 0{\cdot}80$	Contraction
Adrenaline hydrogen tartrate ..	10	24	No change $0{\cdot}20 < P < 0{\cdot}30$	No change $0{\cdot}60 < P < 0{\cdot}70$	No change
,, ,,	100	12	Increased $0{\cdot}01 < P < 0{\cdot}02$	Increased $P < 0{\cdot}001$	No change
,, ,,	100	12*	Increased $0{\cdot}02 < P < 0{\cdot}05$	Increased $P < 0{\cdot}001$	No change
Carbachol chloride	0·3	20	No change $0{\cdot}40 < P < 0{\cdot}50$	No change $0{\cdot}70 < P < 0{\cdot}80$	Contraction
Histamine acid phosphate ..	2	24	No change $0{\cdot}90 < P$	No change $0{\cdot}30 < P < 0{\cdot}40$	Small contraction
5-Hydroxytryptamine creatinine sulphate	50	24	Increased $P < 0{\cdot}001$	No change $0{\cdot}20 < P < 0{\cdot}30$	Small contraction
Papaverine sulphate	10	24	Increased $P < 0{\cdot}001$	No change $0{\cdot}40 < P < 0{\cdot}50$	Relaxation
,,	200	24*	Increased $P < 0{\cdot}001$	Increased $0{\cdot}01 < P < 0{\cdot}02$	Relaxation

*Exposure time 10 min.
All other cases 1 min.

(10 μg/ml) had no significant effect on $^{47}Ca^{2+}$ release, but a large dose (100 μg/ml) increased $^{47}Ca^{2+}$ release significantly without causing any relaxation. Papaverine caused a marked and prolonged relaxation of the depolarized muscle with a significantly increased release of $^{47}Ca^{2+}$. At 4 min 5-hydroxytryptamine significantly increased $^{47}Ca^{2+}$ efflux but had no significant effect at 7 min. A similar pattern was shown by 10 μg/ml papaverine.

During an acetylcholine or carbachol-induced contraction of the depolarized muscle neither we nor Durbin & Jenkinson (1961a) could record a significant increase in calcium efflux, although an increased uptake can be shown (Robertson 1960; Durbin & Jenkinson, 1961a; Banerjee & Lewis, 1964). Schatzmann (1963) has however observed an increased release but no change in uptake of calcium, during stimulation of the non-depolarized taenia coli with acetylcholine or potassium.

Although acetylcholine, carbachol and histamine cause the depolarized muscle to contract, $^{47}Ca^{2+}$ efflux does not significantly increase. This may indicate a failure to mobilize the less freely-exchangeable calcium fractions despite the increase in calcium permeability indicated by increased $^{47}Ca^{2+}$ uptake (Robertson, 1960; Durbin & Jenkinson, 1961a; Banerjee & Lewis, 1964). However at 4 min, 5-hydroxytryptamine causes both a small contraction and a significantly increased $^{47}Ca^{2+}$ efflux, but at 7 min had no significant effect. It may therefore mobilize and cause the loss of one or more of the less readily exchangeable fractions of calcium, and at the same time increase calcium permeability as shown by the increased $^{47}Ca^{2+}$ uptake (Banerjee & Lewis, 1964).

If the presence in the muscle of calcium is essential for maintenance or development of tone—as is suggested by the observation that calcium is essential for contraction in depolarized muscle (Robertson, 1960; Durbin & Jenkinson, 1961b), then calcium may be involved in those reactions which yield the energy for the maintenance of smooth muscle tone. Interference with energy-yielding cellular reactions may thus cause loss of calcium because calcium cannot be utilized and this would be reflected in a decreased calcium uptake (Banerjee & Lewis, 1964) and increased calcium release (Table I). The actions of papaverine might be explained on this basis.

Although we could observe no muscle relaxation, the higher dose of adrenaline caused a significant increase in $^{47}Ca^{2+}$ release. The mechanism here is apparently different from that of papaverine. The calcium released may come mainly from a fraction not involved in maintaining muscle tone in depolarized muscle.

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August 18, 1964

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Book Review

SPECTROPHOTOMETRIC ANALYSIS OF DRUGS INCLUDING ATLAS OF SPECTRA. By Irving Sunshine and S. R. Gerber. Pp. xvii + 235 (including Index). Charles C. Thomas, Springfield, Ill., U.S.A., 1963. $10.50.

The infra-red and ultra-violet absorption spectra of a number of commonly used drugs have been determined and presented in *Spectrophotometric Analysis of Drugs including Atlas of Spectra.* This compilation has been made by a toxicologist and the Coroner from Cleveland, Ohio as an aid in the identification of drugs after isolation from blood, urine or stomach contents. Towards this end a simplified isolation procedure is suggested. It is based on solvent extraction of, for example, blood after precipitation of the proteins by zinc salts in alkaline solution. The method is claimed to be preferable to the classical Stas Otto technique.

The ultra-violet absorption spectra have been recorded on a Beckmann DK2 recording spectrophotometer in both 0·1 N sulphuric acid and 0·1 N sodium hydroxide. Infra-red spectra were run on a Perkin Elmer model 21 in potassium bromide pellets and, where solubility allowed, in chloroform solution. Apart from the statement that "material isolated is pressed into a KBr disc," details of sample handling and recording conditions are totally absent: for example no path length is quoted for the solution spectra. In view of the well known dangers of the potassium bromide technique, some reference to this might have been expected. Within the field of barbiturates alone there are a number of publications drawing attention to the difficulties of such spectra. The authors give no hint of appreciating this point although they reproduce the spectra for about 18 barbiturates.

In one other respect this publication may be faulted. No chemical formulae are given and substances are referred to throughout by what the authors state are "generic names." These are apparently a mixture of trade names, official names and others. For identification purposes the use of an approved name in every case where it existed would have facilitated recognition; structural formulae would certainly have clarified the matter.

In respect of such criticisms the present volume compares badly with the spectra collection of the USP and NF reference standards although of course it covers a wider field. The volume is indexed for 268 infra-red spectra and 143 ultra-violet and visible spectra: it provides no literature references to previous spectroscopic work on any of the substances named therein.

D. W. M.

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