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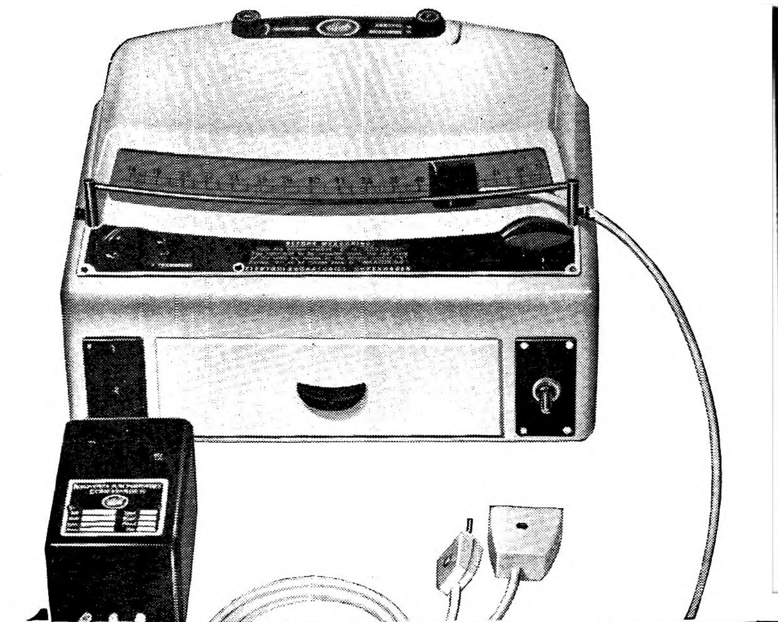

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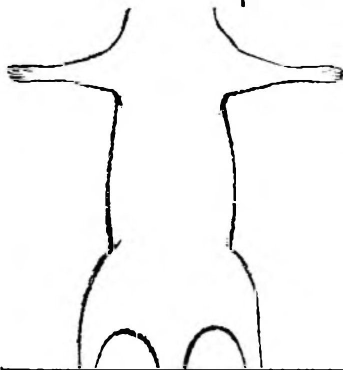

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J. Pharm. Pharmac., 1966, **18**, 561–568 Received April 25, 1966

Modification of drug responses by hydrolytic Enzymes

ALAN W. CUTHBERT

α-Chymotrypsin and phospholipase enzymes have been used to alter the cell membranes of the smooth muscle of the guinea-pig taenia coli. The effects of these treatments on the electrical and mechanical responses of the muscle to acetylcholine have been studied. Prolonged treatment with both enzymes causes depolarization with a consequent loss of acetylcholine sensitivity. After controlled trypsinization a membrane response to acetylcholine was obtained in the absence of a mechanical response. The sensitivity of the taenia coli to acetylcholine was depressed by controlled phospholipolysis without affecting the membrane potential or contractile apparatus. The implications of these findings are discussed.

IT is generally supposed that drug receptors in smooth muscle are specialized protein molecules located on the cell membrane. These are considered to undergo a conformational or other change on reaction with the drug resulting in permeability or other changes which in their turn lead to the mechanical response.

Recently suggestions have been made that a phospholipid component of the cell membrane is implicated in chemoceptive action. Dikstein & Sulman (1965a) found that when rabbit aortic strips were exposed to labelled dibenamine the drug was bound to a cephalin fraction. Further, the binding of dibenamine was prevented by adrenaline. The same authors (Dikstein & Sulman, 1965b) also found that the responsiveness of the frog rectus and rabbit uterus preparations to drugs was reduced by treating with 25% acetone for 2 min and that reactivity in these preparations was restored by treating the preparations with phosphatidylethanolamine or phosphatidylserine. Woolley & Gommi (1964) found that the combined action of neuraminidase and EDTA on rat stomach strips selectively inhibited the action of 5-hydroxytryptamine (5-HT). Sensitivity to this agent was quickly restored by adding a crude extract of stomach lipids and the authors suggested that the 5-HT receptor is a neuraminidase-sensitive ganglioside.

In this paper hydrolytic enzymes have been used to alter the protein or lipid components of the cell membrane (Tobias, 1958) and the effect of this treatment on the responses to drugs, particularly acetylcholine, has been investigated.

Experimental

MATERIALS AND METHODS

The preparation used was the isolated taenia coli of the guinea-pig. The physiological saline solution employed throughout was a Krebs solution of the following composition: (mM) NaCl, 118; KCl, 4·7; $CaCl_2$ 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 25; NaH_2PO_4, 1·1; and glucose, 5·6. Preparations were mounted either in an isolated organ bath maintained at 37° and gassed with a mixture of oxygen 95% and carbon dioxide 5%, or in a sucrose gap electrode at 37°. The latter was of conventional design and provision was made for recording the isometric tension of the preparation simultaneously with the electrical membrane activity. The catecholamine

From the Department of Pharmacology, Downing Street, Cambridge.

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content of guinea-pig hearts was estimated fluorimetrically by a method based on that devised by Euler & Lishajko (1961).

DRUGS AND ENZYMES

Drugs were given in two ways to preparations mounted in the sucrose gap electrode. Either the Krebs solution flowing through the apparatus was changed to Krebs solution containing dissolved drug or the drug was injected, in a small volume of Krebs solution, into the fluid perfusing the tissue. The following two enzymes were used; α-chymotrypsin (EC 3.4.4.5, Seravac Laboratories Ltd., salt free with an activity of 11,000 ATEE u/mg) and phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3, Koch-Light Laboratories Ltd., from *Clostridium welchii*).

Results and discussion

When guinea-pig taenia coli, mounted in an organ bath, were exposed to phospholipase C or to α-chymotrypsin the responses of the muscle strips to acetylcholine were eventually abolished. In the case of the phospholipase enzyme, a 2 hr exposure to a concentration of 20 μg/ml was sufficient to abolish the response to acetylcholine. The reduction in the sizes of the responses during exposure to the enzyme was gradual and showed no unusual features. In some experiments there was a small increase in tone of the muscle strip following addition of the phospholipase. With α-chymotrypsin (0·5 or 1·0 mg/ml) the results were more complicated. The enzyme itself caused a large increase in tone which then declined, during 1–2 hr, to a level lower than that existing before exposure to the enzyme. During the first hour of exposure the response to acetylcholine was still present, although reduced during the period of raised tone. Then, suddenly, addition of acetylcholine caused the muscle to relax. These inhibitory responses could be obtained three or four times during 20 min and showed tachyphylaxis. Eventually, as with phospholipase C, after about 2 hr no response was obtained to acetylcholine. Inhibitory responses to acetylcholine after exposure to chymotrypsin were not obtained in preparations taken from guinea-pigs given 5 mg/kg reserpine 18 hr previously. The hearts from such animals were found to be 95% depleted of catecholamines and it is concluded that the inhibitory responses of the taenia to acetylcholine were the result of catecholamine release.

The experiments described above were repeated with taenia coli preparations mounted in a sucrose gap electrode so that both tension and electrical membrane responses could be examined. In these results the membrane potential of the tissue is taken as the difference in potential between the active part of the tissue, perfused in warmed Krebs solution at 37°, and the inactive part, perfused in either isotonic K_2SO_4 or isotonic KCl. Such a measure is inaccurate due to the various junction potentials existing between Krebs solution and sucrose, sucrose and K_2SO_4 or KCl, and between the KCl agar of the recording electrodes and the solutions with which they are in contact. These junction potentials may be large

but only a fraction of them will be recorded depending on the short circuiting factor of the gap, that is, the relative impedences offered by tissue compared with the surrounding sucrose solution. In spite of these drawbacks, changes in membrane potential as a result of enzymic and drug treatment will be true as the contribution of the junction potentials to the measured potential will be constant. It is for these reasons that changes in membrane potential have been stated rather than values of the potential.

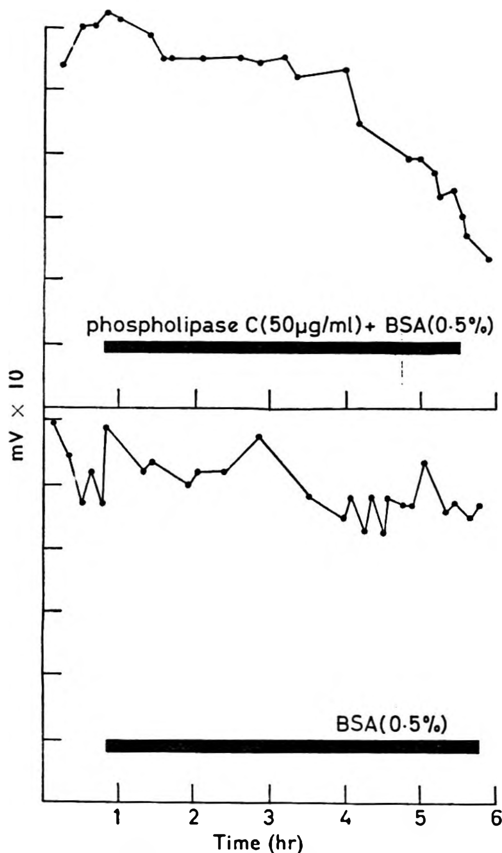

FIG. 1. Changes in the membrane potential of taenia coli smooth muscle strips exposed to phospholipase C (50 μg/ml) + 0·5% Bovine Serum Albumin (BSA) and 0·5% BSA alone, for the periods indicated by the horizontal bars.

Higher concentrations and longer exposures to phospholipase C were required to abolish the response to acetylcholine when taenia coli were placed in the sucrose gap electrode rather than in an organ bath. This is undoubtedly due to inactivation of the enzyme during its flow through the narrow bore tubes of the apparatus. It is known that the enzyme is inactivated at interfaces and particularly by bubbling (Macfarlane & Knight, 1941). Inactivation was kept to a minimum by vigorously bubbling the Krebs solution with oxygen 95% and carbon dioxide 5%

before dissolving the enzyme and by sometimes adding 0·5% bovine serum albumin (BSA) to the solution.

Fig. 1 shows the changes in membrane potential caused by exposure of a muscle strip to phospholipase C (50 μg/ml) for 6 hr. The membrane potential remained steady for the first 4 hr after which rapid depolarization took place, as would be expected if there was a sudden collapse of the membrane structure. The total fall in membrane potential was around 35 mV so that the muscle strip was 70% depolarized, assuming a resting membrane potential of 50 mV (Burnstock & Prosser, 1960). A control preparation exposed to exactly the same conditions but without phospholipase showed only a minor reduction in resting membrane potential in 6 hr (Fig. 1). Acetylcholine, which normally produces a discharge

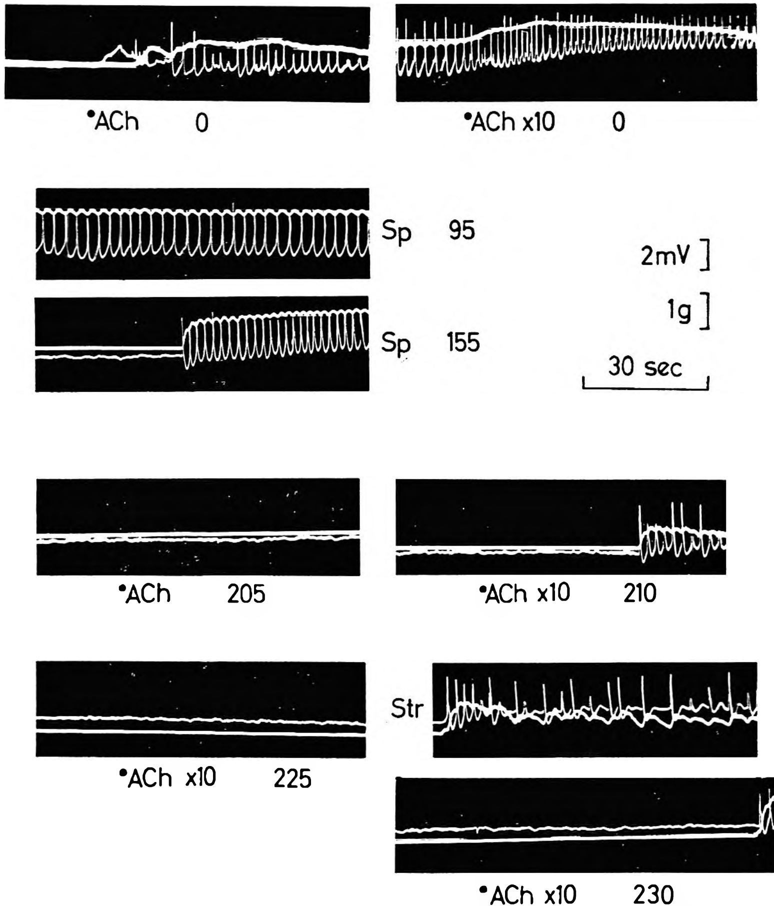

FIG. 2. Electrical and mechanical activity of a guinea-pig taenia coli preparation. The figures by each panel refer to the time, in min, during which the preparation was exposed to phospholipase C (30 μg/ml). At ACh the preparation was exposed to 0·1 μg/ml acetylcholine for 15 sec, at ACh × 10 the concentration was 1·0 μg/ml. Sp refers to spontaneous activity and Str refers to a stretch sufficient to raise the tension 0·1 g.

of action potential spikes, membrane depolarization and a sharp rise in tension was ineffective on preparations which had been depolarized by 3·5–4 hr treatment with phospholipase C. On normal preparations, potassium ions produced responses qualitatively similar to acetylcholine but in the phospholipase-treated preparation potassium ions produced only a residual depolarization unaccompanied by any spike potentials. On the other hand, caffeine (1 mg/ml) produced a contractile response in phospholipase treated muscles without any membrane response. This is in accord with the idea that caffeine liberates bound calcium from the cell membrane (Herz & Weber, 1965) and that this occurs just as well in depolarixed tissues (Axelsson & Thesleff, 1958). As might be expected the phospholipase depolarized muscles were insensitive to a stretch stimulus. Control preparations which had not been subjected to phospholipase treatment responded normally to drugs and stretch 5 hr after mounting in the apparatus.

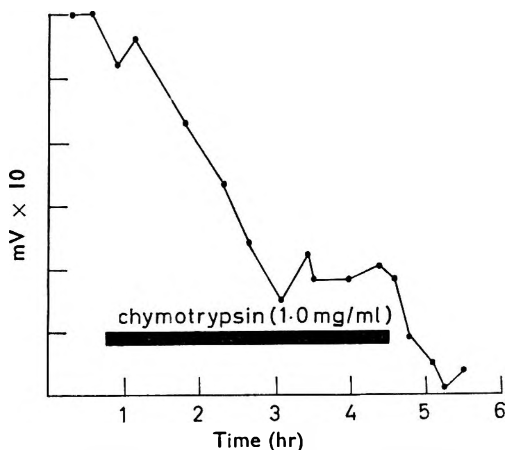

FIG. 3. Changes in the membrane potential of a taenia coli smooth muscle strip exposed to α-chymotrypsin (1 mg/ml) for the period indicated by the horizontal bar.

So far, the consequences of depolarization by phospholipase C are predictable and of little interest. However, by controlled phospholipase treatment, effects on drug responses were obtained at a time at which the membrane remained polarized. This phenomenon is illustrated in Fig. 2. Responses of a taenia strip to two concentrations of acetylcholine were determined after which phospholipase C (30 μg/ml) was added to the perfusion fluid. The preparation was spontaneously active, at first continuously and then intermittently. This is illustrated by recordings taken after 95 and 155 min exposure to phospholipase C. After some 200 min exposure to the enzyme bursts of spontaneous activity were less frequent but the membrane potential remained normal. The measured potential difference being 60 mV after 200 min exposure to the enzyme compared with values ranging from 45 to 58 mV at the beginning of the experiment. The responses to acetylcholine after 200 min of enzyme treatment are shown in Fig. 2, where it can be seen that no response was

obtained to the low concentration and a delayed response to the high drug concentration. It is impossible to tell whether this is really a delayed response or a burst of spontaneous activity commencing 45 sec after the application of the drug. After 225 min and 230 min of enzyme treatment no response was obtained even to the higher concentration of acetylcholine. At this time however the membrane was still excitable as shown by the burst of electrical and mechanical activity caused by applying a slight stretch sufficient to raise the tension in the muscle by 0·1 g.

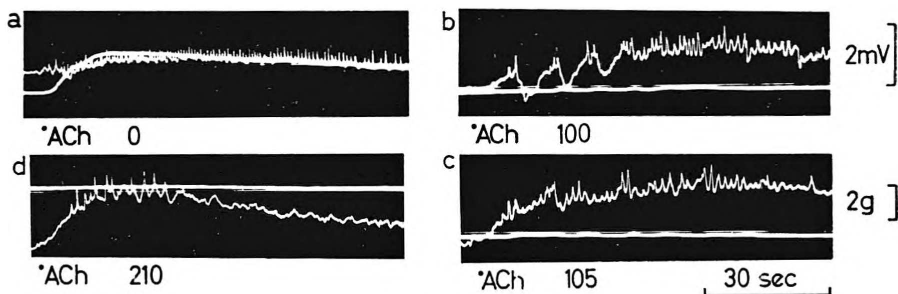

FIG. 4. Electrical and mechanical activity of a guinea-pig taenia coli preparation. At ACh in (a), (b) and (c) the preparation was exposed to acetylcholine (1 μg/ml) for 10 sec and at ACh in (d) to 10 μg/ml for 10 sec. The figures below each panel refer to the time, in min, after which treatment of the preparation with α-chymotrypsin (1 mg/ml) was commenced. The preparation was returned to normal Krebs solution after it had been perfused with the enzyme for 100 min. Note in (d) the tension on the preparation has been raised.

Turning to the effects of α-chymotrypsin on the taenia coli preparation, it was again found that prolonged exposure of the tissue to the enzyme resulted in depolarization with a consequent loss of sensitivity to drugs. Fig. 3 shows the change in membrane potential caused by exposure to α-chymotrypsin (1 mg/ml) for 4 hr. As can be seen the membrane potential fell continuously over this period and continued to do so after the enzyme was removed. The responses of the tissue became progressively more feeble as depolarization proceded. When the membrane potential had fallen by about 40 mV the responses to acetylcholine or potassium ions consisted only of a few isolated action potentials. After a further fall in membrane potential no further responses could be elicited.

As with phospholipase, interesting and unpredictable results were obtained by controlled treatment with α-chymotrypsin. Treating taenia coli with α-chymotrypsin for periods shorter than those required to significantly reduce the membrane potential, abolished the mechanical response without much effect on the membrane changes. This type of result is illustrated in Fig. 4. In this instance the preparation was treated with α-chymotrypsin for 100 min and then returned to normal Krebs solution. Before treatment, acetylcholine produced the usual electrical and mechanical responses but at the end of, and after enzyme treatment, identical acetylcholine concentrations produced only an action potential discharge and depolarization without mechanical response. It is obvious from Fig. 4 that the nature of the electrical discharge has changed after enzyme

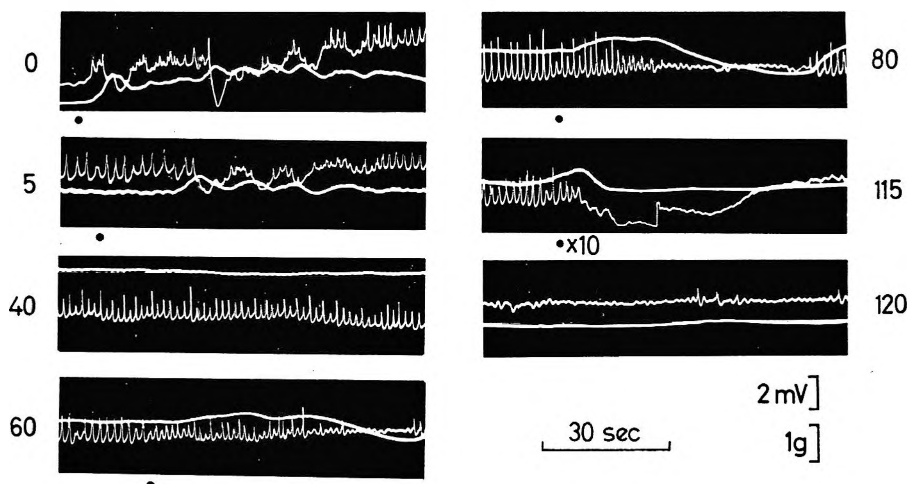

FIG. 5. Electrical and mechanical activity of a guinea-pig taenia coli preparation showing the inhibitory response to acetylcholine occurring during treatment with α-chymotrypsin (0·5 mg/ml). The duration of perfusion with α-chymotrypsin is shown (in min) at the side of each panel. At each dot the preparation was exposed for 15 sec to acetylcholine at a concentration of 1 μg/ml or 10 μg/ml (× 10). Note that this latter dose caused cessation of activity for 5 min. Following this dose the response to acetylcholine reverted to excitation.

treatment, the spikes having a slower rate of rise and fall. However this technique is not an appropriate tool for the study of spike configuration and it must suffice to point out that the character of the discharge was abnormal. It was a possibility that the enzyme treatment had so disorganized the tissue that it was no longer under an appropriate tension to show a mechanical response. Accordingly, the tension was artificially raised, but the same result was obtained (Fig. 4d). In other experiments the same uncoupling phenomenon was shown for potassium ions. From the results with acetylcholine it can be concluded that at least some muscarinic receptors were intact and functional after enzyme treatment although the normal contraction was not obtained.

As was pointed out earlier, some preparations responded to acetylcholine during α-chymotrypsin treatment with a relaxation rather than a contraction. The genesis of this effect is illustrated in Fig. 5. After 60 min tryptic digestion a biphasic response to acetylcholine was obtained, and similarly at 80 and 115 min with the inhibitory effect becoming more pronounced. The inhibition of spike activity, hyperpolarization and relaxation seen in Fig. 5 are typical of the response of the taenia coli to catecholamines and it is considered that acetylcholine is here acting by releasing catecholamines, probably from adrenergic nerves. Acetylcholine is thought to release catecholamines from adrenergic nerves (see Burn & Rand, 1965 for references) and it would appear that trypsinization of the taenia coli facilitates this. Fig. 5 also illustrates the increase in tone and electrical discharge caused by α-chymotrypsin alone. Trypsin produces a contracture in many smooth muscles and is thought to do so by the release of spasmogenic polypeptides or histamine or both (Rocha

e Silva, 1956). The stimulatory effect of α-chymotrypsin may result from a similar mechanism.

Conclusions

As was anticipated, this study has not been able to prove the chemical nature of the muscarinic receptors in smooth muscle. Two unexpected findings however emerge from the results. First, the muscarinic receptor is sufficiently resistant to tryptic digestion to remain excitable at a time when the contractile response has been abolished. Second, controlled phospholipolysis inhibits the action of acetylcholine while the membrane and contractile apparatus remain functional. Each finding is capable of many interpretations. The first may be interpreted thus: (i) the acetylcholine receptor is not protein, (ii) the acetylcholine receptor is protein but is not hydrolysed by α-chymotrypsin, (iii) α-chymotrypsin has attacked the coupling mechanism, (iv) the configurations of the action potentials are so changed that they no longer activate the coupling mechanism and (v) the contractile apparatus is inactivated. The second finding may be interpreted to mean that (i) the acetylcholine receptor is a phospholipid or (ii) phospholipids are involved in coupling receptor activation to the permeability change of the membrane.

It is known that agonist drugs, like acetylcholine, can cause enormous changes in ion permeability in smooth muscle, including the taenia coli (Burgen & Spero, 1966). It seems impossible that these almost explosive changes do not involve the lipids of the membrane if the Danielli-Davson (1935) concept of the cell membrane is accepted. Large permeability changes would seem to require a change in membrane ultrastructure with the creation of pores through which the lipid-insoluble ions can pass. This work has shown that the excitation of smooth muscle by acetylcholine is dependent on the integrity of some membrane lipids, as indicated by the decreased sensitivity shown to acetylcholine by preparations treated with phospholipase C. What is not known is whether drugs, like acetylcholine, affect a conformational change in the membrane protein which then causes a second order rearrangement of the membrane lipids or whether the drug interacts directly with the lipids. This vital question remains to be answered.

References

Axelsson, J. & Thesleff, S. (1958). *Acta physiol. scand.*, **44**, 55–66.
Burgen, A. S. V. & Spero, L. (1966). Communication to British Pharmacological Society, Liverpool.
Burn, J. H. & Rand, M. J. (1965). *Ann. Rev. Pharmac.*, **5**, 163–182.
Burnstock, G. & Prosser, C. L. (1960). *Am. J. Physiol.*, **199**, 553–559.
Danielli, J. F. & Davson, H. (1935). *J. cell. comp. Physiol.*, **5**, 495–508.
Dikstein, S. & Sulman, F. G. (1965a). *Biochem. Pharmac.*, **14**, 881–885.
Dikstein, S. & Sulman, F. G. (1965b). *Ibid.*, **14**, 739–742.
Euler, U. S. von & Lishajko, F. (1961). *Acta physiol. scand.*, **51**, 348–355.
Herz, R. & Weber, A. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24**, 208.
Macfarlane, M. G. & Knight, B. C. J. G. (1941). *Biochem. J.*, **35**, 884–902.
Rocha e Silva, M. (1956). In *Histamine, Ciba Foundation Symposium*, p. 124–138, London: Churchill.
Tobias, J. M. (1958). *J. cell. comp. Physiol.*, **52**, 89–125.
Woolley, D. W. & Gommi, B. W. (1964). *Nature, Lond.*, **202**, 1074–1075.

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The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of *Escherichia coli* and *Staphylococcus aureus*

W. B. HUGO AND A. R. LONGWORTH*

Chlorhexidine does not cause lysis of isolated cell walls, nor does it prevent the synthesis of the mucopeptide component of the cell wall. Low concentrations of the drug stimulate dehydrogenase activity but higher concentrations inhibit the activity. Chlorhexidine reacts with and precipitates proteinaceous and pentose-containing components of a solution of cell-free cytoplasmic constituents in concentrations greater than those causing their maximum leakage. The effect of chlorhexidine concentration on the electrophoretic mobility of bacterial cells is consistent with the hypothesis that the drug accumulates in aggregates at the cell surface rather than in the form of a monolayer or multilayers of drug.

IT has been shown that chlorhexidine causes the release of cytoplasmic constituents from bacterial cells (Hugo & Longworth, 1964a; Rye & Wiseman, 1964; 1965), presumably by damaging the cytoplasmic membrane of the cell. The nature of the disruptive reaction and the cause of the apparent inhibition of leakage have been examined and an attempt made to correlate leakage with inactivation of dehydrogenase activity (Hugo, 1954). The effect of chlorhexidine on the electrophoretic mobility of bacterial cells has also been investigated.

Experimental

MATERIALS

Materials, conditions of culture, preparation of bacterial suspensions and, wherever possible, suspension densities were the same as those previously used (Hugo & Longworth, 1964a). The medium used in the determination of the accumulation of *N*-acetylamino-sugars was yeast extract 5 g, peptone 5 g, dipotassium hydrogen phosphate 1 g, distilled water to 1 litre at pH 7·2.

Unless otherwise stated experiments were made in duplicate.

Effect of chlorhexidine on isolated bacterial cell walls. Isolated bacterial cell walls were obtained by disruption of bacterial cells in a Mickle tissue disintegrator (Mickle, 1948) with ballatoni beads (No. 15 average diameter 0·1 mm) as described by Salton & Horne (1951). Maximal disruption of *E. coli* was obtained by shaking 10 ml of cell suspension (12 mg dry weight cell/ml) for 90 min with 12 g of beads at 4°. For *Staph. aureus* maximal disintegration was achieved after 150 min agitation at 4° of 10 ml of cell suspension (5 mg dry weight cells/ml) with 6 g of beads. After disintegration of the cells, the glass beads were removed on a No. 3 sintered glass filter and the filtrate diluted with distilled water. Intact cells were removed from the filtrate by centrifugation (2,000 × *g* for 10 min), the supernatant liquid was decanted and recentrifuged (10,000 × *g* for 15 min) to recover cell walls. The walls so obtained were washed

From the Department of Pharmacy, Nottingham University.

* Present Address: School of Pharmacy, Leicester Regional College of Technology.

three times with phosphate buffer (0·2 M pH 7·0) to remove adhering cytoplasmic contents and suspended in distilled water. Dry weight determinations were made on the original bacterial suspensions and on the cell wall suspensions.

The percentage of the dry weight of cells obtained as walls was 27·5 for *E. coli* and 20·6 for *Staph. aureus*. The preparations were examined by interference microscopy and were found to be free from intact cells.

Lysis of bacterial cell walls by chlorhexidine. Changes in absorbance at 500 mμ of suspensions of cell walls of *E. coli* and *Staph. aureus*, 0·332 and 0·306 dry weight cells/ml respectively, in aqueous solutions of chlorhexidine, 0–800 μg/ml, at 20° were determined over a period of 12 hr in 1 cm glass cuvettes and were read against a reference cuvette containing a suspension of cell walls in water.

Effect of chlorhexidine on cell wall synthesis. Penicillin has been shown to cause an accumulation of *N*-acetylamino-sugars in growing cultures of *Staph. aureus* (Strominger, 1957). This accumulation is believed to indicate impaired synthesis of the rigid (mucopeptide) component of bacterial cell walls. In media where the osmotic pressure is less than that of the cytoplasm this accumulation of sugars is followed by cell lysis.

It has been shown that chlorhexidine also causes the leakage of cytoplasmic constituents from bacterial cells (Hugo & Longworth, 1964a). The effects of chlorhexidine and penicillin on the accumulation of *N*-acetylamino-sugars were compared.

Five ml of an overnight culture of *Staph. aureus* in yeast extract medium was added to 500 ml of yeast extract medium in conical culture flasks and incubated at 37°. After 6 hr incubation the cell suspension density of the culture was determined nephelometrically. Reference was made to a calibration curve prepared by dilution of standard aqueous suspensions of *Staph. aureus* with an equal volume of double strength yeast extract medium. The cultures were then treated variously with 1 ml volumes of sterile water, benzylpenicillin, 500 μg/ml (sodium salt 1,667 u/mg), or chlorhexidine, 500 μg/ml or 5,000 μg/ml. After a further 90 min incubation, the opacity of the suspension was redetermined and the cells from 400 ml of medium harvested by centrifugation. The cells were resuspended in water and again harvested and the accumulation of *N*-acetylamino-sugars (calculated as *N*-acetylglucosamine) determined by the method of Strominger (1957).

The effect of chlorhexidine on the reduction of 2,3,5-*triphenyltetrazolium bromide* (TTB) *by bacterial cells.* Washed suspensions of *E. coli* and *Staph. aureus* containing 2·4 mg dry weight cells/ml in water were prepared and allowed to equilibrate at 37° for 1 hr before use. To 10 ml glass centrifuge tubes, 0·5 ml of 0·13 M phosphate buffer pH 7·3, 1 ml of a solution of TTB, 250 μg/ml, and 1 ml of water or chlorhexidine solutions were added. The tube contents were allowed to equilibrate to 37° and 2·5 ml of bacterial suspension added to each tube such that the final suspension contained 1·2 mg dry weight cells/ml in 0·013 M phosphate buffer, 50 μg/ml TTB and various concentrations of chlorhexidine.

After thorough mixing the cultures were incubated in a water-bath at 37° for 45 min, 5 ml volumes of acetone were added to each tube to extract the coloured formazan produced by reduction of TTB, the cells were removed by centrifugation (5,000 × *g* for 10 min) and the absorbance of the acetone solution containing the formazan determined spectrophotometrically at 525 mμ. No increase in absorbance at 525 mμ was noted in control experiments which included cells held at 100° for 10 min before addition to the TTB/buffer system or in other controls lacking TTB or cells.

Reaction of chlorhexidine with cell-free cytoplasmic constituents of bacteria. Cell suspensions of *E. coli* and *Staph. aureus* were disintegrated in a Mickle tissue disintegrator at 4° as previously described. After removal of the glass beads and whole cells by centrifugation at 5,000 × *g* for 20 min, the supernatant solution was diluted with distilled water such that the cytoplasmic constituents contained in 1 ml of solution were derived from 2·4 mg dry weight of cells. The pH values of the supernatant solutions of *E. coli* and *Staph. aureus* were 6·8 and 7·3 respectively.

To 5 ml of the solution of cytoplasmic constituents, 5 ml amounts of aqueous solutions of chlorhexidine were added. After 10 min contact at 20° the solutions were recentrifuged (5,000 × *g* for 10 min) and the supernatant solution decanted. The amounts of protein and nucleic acid present in the supernatant solutions were determined as ninhydrin-positive material (Kabat & Mayer, 1961) and pentoses (Mejbaum, 1939) respectively. Control experiments were included in which the amounts of protein and pentoses, in the solution of cytoplasmic constituents diluted with an equal volume of water, were determined before and after centrifugation. This was to find the concentration of protein and nucleic acid present and to show that no precipitation of cytoplasmic constituents occurred on dilution with water.

The uptake of chlorhexidine by the solution of cytoplasmic constituents was determined by colorimetric analysis (Holbrook, 1958) of the supernatant solution after removal of the precipitate. The absorbance values were corrected for the turbidity of the supernatant solution.

The percentages of pentoses and proteins which were precipitated by treatment with various concentrations of chlorhexidine were then calculated.

Effect of chlorhexidine on the electrophoretic mobility of bacterial cells. The mobility of a particle under the influence of an applied potential depends on several factors including the magnitude of the applied potential and also the nature of the surface of the particle; this latter factor is, in turn, influenced by the ionic strength and pH of the suspending medium. Hence changes in electrophoretic mobility in a medium of constant ionic strength and under constant potential will reflect changes in the nature of the surface.

On the basis of earlier work (Hugo & Longworth, 1964a), it appears that chlorhexidine is bound at the surface of bacteria and an examination was made of the electrophoretic mobility of bacterial cells treated with chlorhexidine. This was designed to assess the effect of the compound

on the magnitude of the surface charge and on the electrophoretic homogeneity of the population.

A horizontal cylindrical cell of internal diameter 3 mm and length 12 cm was calibrated using an erythrocyte suspension. All observations were made in the stationary layer. Silver/silver chloride electrodes were used. The potential gradient down the cell was between 5 and 10 V/cm calculated as recommended by Moyer (1936) from the specific resistance of the bacterial suspension, measured directly in the electrophoresis cell, and from the current flowing. The apparatus was based on that of McQuillen (1952); the construction and operation of the apparatus are described by Longworth (1965).

The procedure adopted was to mix equal volumes of bacterial suspensions, 0·2 mg dry weight cells/ml in phosphate buffer ionic strength (I) 0·02, pH 7·3, at 20° with aqueous solutions of chlorhexidine. The suspension was then run into the micro-electrophoresis cell and the first mobility measurement on the suspension (0·1 mg dry weight cells/ml in phosphate buffer; I = 0·01, pH 7·3) containing various concentrations of chlorhexidine was made 10 min after contact. The times taken by 20 individual bacterial cells to travel a distance of 105 μ with the current passing first in one and then in the other direction were measured. A set of observations was completed in 10 min and from the average velocity and the potential gradient the electrophoretic mobility (μ/sec/V/cm) was calculated.

The adsorption of chlorhexidine by bacterial cells (0·1 mg dry weight cells/ml in phosphate buffer, I = 0·01, pH 7·3 at 20°) after 10 min contact was determined (Hugo & Longworth, 1964a).

Results

Lysis of bacterial cell walls by chlorhexidine. There was no spectrophotometric evidence of dissolution of cell walls. An increase in extinction of cell wall suspensions with increase in chlorhexidine concentration was observed. This possibly corresponded to increased light scattering properties of the walls caused by adsorbed chlorhexidine (Hugo & Longworth, 1964a).

Accumulation of N-*acetylamino-sugars.* The effects of chlorhexidine and penicillin on the accumulation of *N*-acetylamino-sugars are shown in Table 1.

TABLE 1. ACCUMULATION OF *N*-ACETYLAMINO-SUGARS BY *Staph. aureus* CELLS IN THE PRESENCE OF PENICILLIN AND CHLORHEXIDINE

Treatment	Dry wt of cells (mg/ml) determined nephelometrically		μmoles N-acetyl glucosamine/mg dry weight of cells after 7½ hr incubation
	before treatment i.e. after 6 hr incubation	after treatment i.e. after 7½ hr incubation	
None	0·036	0·082	0·005
Penicillin, 1 μg/ml	0·036	0·052	0·01
Chlorhexidine, 1 μg/ml	0·036	0·07	0·0023
Chlorhexidine, 10 μg/ml	0·036	0·036	0·0002

Cells grown for 90 min in the presence of a concentration of penicillin which impairs growth show an increased level of *N*-acetylamino-sugars over untreated cells. Concentrations of chlorhexidine which impair (1 μg/ml) and inhibit (10 μg/ml) growth under the conditions of the test cause no such accumulation.

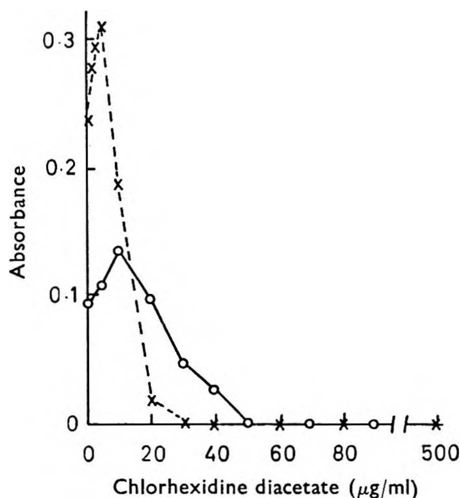

FIG. 1. Effect of chlorhexidine diacetate on the absorbance at 525 mμ of the supernatant solution derived from a suspension of *E. coli* —○—○— and *Staph. aureus* ×- - - - -× (1·2 mg dry weight of cells/ml in 0·013 M phosphate buffer containing 50 μg/ml TTB) by dilution with an equal volume of acetone and removal of cells.

Reduction of 2,3,5-*triphenyltetrazolium bromide.* Fig. 1 shows the effect of chlorhexidine concentration on the reduction of TTB by *E. coli* and *Staph. aureus* suspensions. Low concentrations of chlorhexidine stimulate reduction of TTB by bacterial cells whilst high concentrations inhibit the reduction. Reference to the results of experiments assessing the leakage of cytoplasmic constituents from *E. coli* and *Staph. aureus* cells treated with chlorhexidine (Hugo & Longworth, 1964a) shows that the concentration of chlorhexidine which causes maximum leakage also inhibits TTB reduction. On the other hand lower concentrations, which cause a slight leakage, stimulate TTB reduction.

Precipitation of cytoplasmic constituents. The effects of chlorhexidine on precipitation of cell-free cytoplasmic constituents derived from *E. coli* and *Staph aureus* cells are shown in Figs 2A and B.

Low concentrations of chlorhexidine, from 0–150 μg/ml, cause no precipitation of the proteins and nucleic acid derived from *E. coli* cells. Higher concentrations precipitate approximately 90% of the nucleic acids and approximately 50% of the proteins present. Chlorhexidine is removed from solution presumably by precipitation with cytoplasmic constituents. The concentration of chlorhexidine which causes maximum leakage from *E. coli* cells detected biochemically and observed cytologically (Hugo & Longworth, 1964a, 1965) causes no precipitation. Higher

concentrations, which inhibit leakage and produce a cytoplasm having a coagulated appearance in electron micrographs, precipitate cytoplasmic constituents. For *Staph. aureus* a similar correlation exists between biochemically detectable leakage and precipitation of cytoplasmic constituents.

Precipitation of cytoplasmic constituents and inhibition of leakage of cytoplasmic constituents from whole cells (Hugo & Longworth, 1964a) is achieved at a lower concentration of chlorhexidine with *Staph. aureus* than with *E. coli*.

Increasing the time of contact between the solution of cytoplasmic constituents and chlorhexidine from 10–120 min had no effect upon the amount of material precipitated.

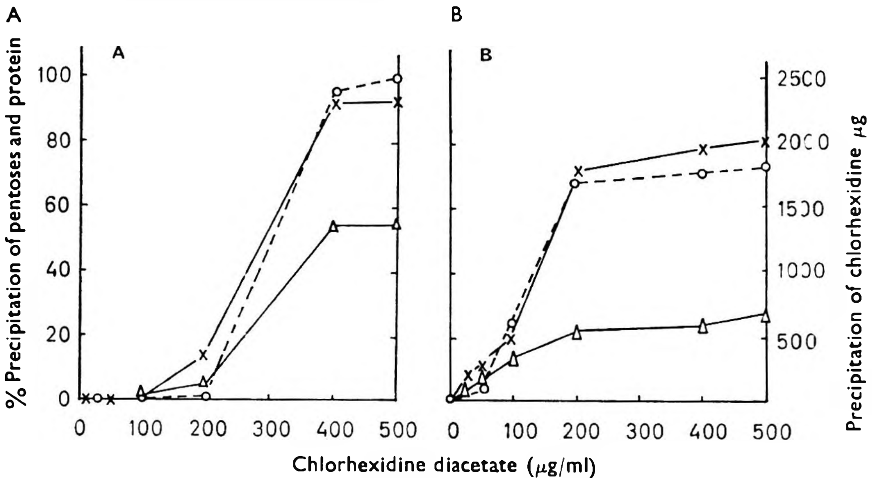

FIG. 2. Effect of chlorhexidine concentration on the precipitation of cytoplasmic constituents derived from 1·2 mg dry weight cells/ml after 10 min contact at 20°. A. *E. coli*. B. *Staph. aureus*. ×—× precipitation of pentoses, %. △—△ precipitation of proteins, %. ○- - - - -○ precipitation of chlorhexidine.

Electrophoretic mobility. The effect of chlorhexidine concentration on the electrophoretic mobility of *E. coli* and *Staph. aureus* cell suspensions and the adsorption of chlorhexidine is shown in Figs 3A and B respectively.

Chlorhexidine causes a decrease in the electrophoretic mobility of both species of organism, and a relationship was found between the amount of chlorhexidine bound by the cells and the mobility. With *Staph. aureus* the charge on the cell was not reversed and the cells retained an overall negative charge in high concentrations of chlorhexidine. The mobilities of *E. coli* cells in the presence of 400–600 µg/ml chlorhexidine were too low for accurate measurements but the cells retained a negative charge. At 600 µg/ml, although the mobility was still very low, the cells had become positively charged. The adsorption of chlorhexidine (µg/mg dry weight of cells) by *E. coli* was in excess of that observed in previous experiments.

Agglutination of suspensions of *E. coli* which occurred in high concentrations of chlorhexidine could explain the reversal of charge in concentrations greater than 600 μg/ml and increased uptake in terms of non-specific absorption of chlorhexidine into the interstices of agglutinated cells.

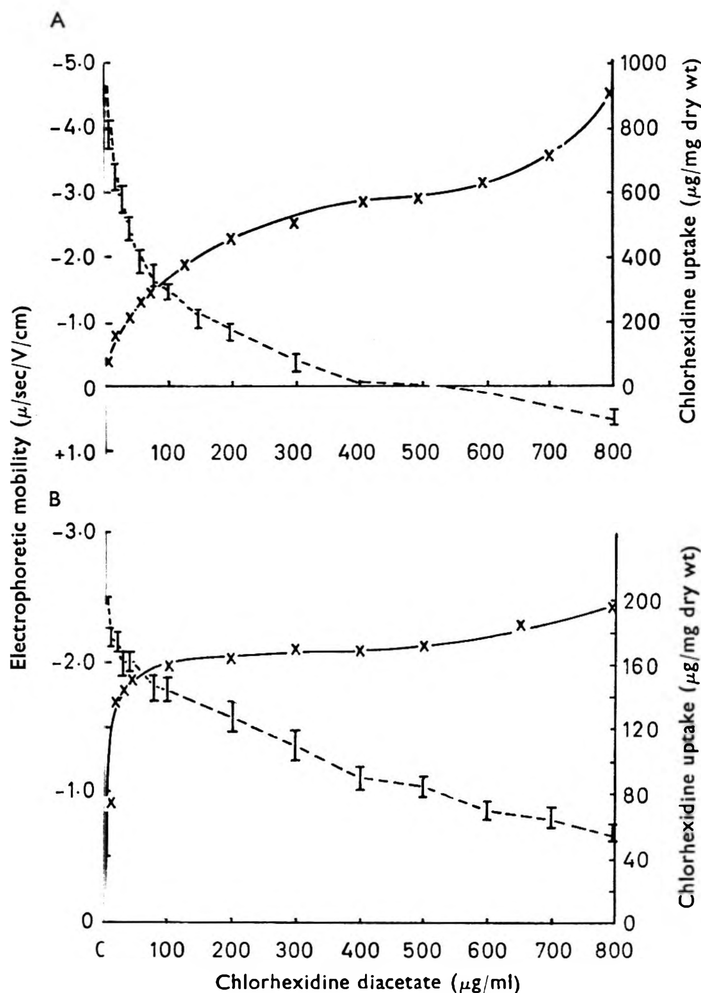

FIG. 3. Effect of chlorhexidine diacetate concentration on the electrophoretic mobility of bacterial cells, 0·1 mg dry weight cells/ml in phosphate buffer (I = 0·01 ; pH 7·3) and the adsorption of chlorhexidine after 10 min contact at 20°. A. *E. coli*. B. *Staph. aureus*. - - - - - electrophoretic mobility. ×—× adsorption of chlorhexidine μg/mg dry weight of cells.

It has been calculated that the maximum amount of chlorhexidine which can be bound in the form of a monolayer at the surface of *E. coli* cells is 85·5 μg chlorhexidine diacetate/mg dry weight cells (Hugo &

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Longworth, 1964a). From Fig. 3 it can be seen that at this level of adsorption the cells are negatively charged. This suggests that a complete layer of chlorhexidine is not formed at the cell surface.

Discussion

Chlorhexidine causes the release of cellular constituents from *Micrococcus lysodeikticus* (Rye & Wiseman, 1964) and from *E. coli* and *Staph. aureus* (Hugo & Longworth, 1964a). This initial leakage is followed, in the presence of low concentrations of chlorhexidine, by a secondary release. In the presence of high concentrations of the drug this secondary release is inhibited (Hugo & Longworth, 1964a; Rye & Wiseman, 1965). Mean single survivor time data indicate that chlorhexidine is more effective as a bactericide at concentrations which inhibit the secondary release (Hugo & Longworth, 1964a). Chlorhexidine causes lysis of "protoplasts" and spheroplasts of *E. coli* and prevents the transformation by lysozyme of *Bacillus megaterium* cells to protoplasts (Hugo & Longworth, 1964b). These results strongly suggest that chlorhexidine causes a disruption of the cytoplasmic membrane.

Chlorhexidine does not dissolve (lyse) isolated cell walls and unlike penicillin, bacitracin and novobiocin does not produce an intracellular accumulation of precursors of the rigid mucopeptide component of the cell wall. With concentrations of chlorhexidine which cause leakage of cytoplasmic constituents, the leakage is immediately detectable (Hugo & Longworth, 1964a). This suggests that membrane disruption is a direct effect of chlorhexidine action rather than a secondary effect of cell wall lysis or impaired cell wall synthesis. Molecular orientation of the drug adsorbed at the cell surface, possibly in a lipid component of the cytoplasmic membrane, could therefore be causing a disorientation of the membrane structure and consequent leakage of the cytoplasmic constituents. Support for this is afforded by experiments on the behaviour of chlorhexidine at the oil-water interface (Hugo & Longworth, 1964a).

The failure of chlorhexidine to neutralize the charge on *E. coli* cells in concentrations producing a level of drug adsorption several times greater than the amount required to form a monolayer around the cells, suggests that chlorhexidine is not adsorbed in the form of a monolayer. Observation of electron micrographs of *E. coli* cells treated with 600 μg/ml chlorhexidine for 10 min revealed the presence of surface protuberances observed in previous work with high concentrations of chlorhexidine (Hugo & Longworth, 1965).

The rapid production of surface swellings and the inability of chlorhexidine to cause cell wall lysis or impair cell wall synthesis suggests that these surface swellings represent local accumulation of chlorhexidine at the cell surface. Such an accumulation of aggregates of chlorhexidine molecules rather than the formation of layers of the agent would explain its inability to neutralise the negative charge on the cell surface. Giles & McKay (1965) conclude from adsorption studies that aggregation of basic dyes occurs at the surface of formalin fixed yeast cells.

In Table 2 results for *E. coli* reported in this paper are correlated with those obtained earlier (Hugo & Longworth, 1964a,b, 1965). The correlation of precipitation of cell-free cytoplasmic constituents with the effect of chlorhexidine on biochemically detectable and cytologically observable leakage of cytoplasmic constituents, supports the suggestion that chlorhexidine in high concentrations inhibits leakage by causing precipitation of cytoplasmic constituents. The observed stimulation of TTB reduction can be interpreted in terms of increased penetration of

TABLE 2. SUMMARY OF RESULTS OBTAINED FOR THE ACTION OF CHLORHEXIDINE ON *E. coli*

Reaction concentration µg/ml chlorhexidine diacetate	Adsorption of chlorhexidine µg/mg dry wt *E. coli* cells (*a*)	Electrophoretic mobility µ/sec/V/cm	Leakage mg dry wt cells after 6 hr contact		Bactericidal activity mean single survivor time min (*a*)
			E at 260 mµ (*a*)	Pentoses µg (*a*)	
0	0	4·62	0·04	0·8	—
5	5	4·52	0·09	1·4	—
10	10	4·40	0·14	2·0	—
90	72	3·40	0·53	9·2	3981
200	160	2·50	0·175	3·6	417
500	293	1·55	0·12	3·4	33

Reaction concentration µg/ml chlorhexidine diacetate	Reduction of tetrazolium as % of reduction by untreated cells	Electron microscopic appearance after 6 hr treatment (*b*)	% precipitation of cell free cytoplasmic constituents	
			protein	nucleic acid
0	100	"normal"	0	0
5	110	—	0	0
10	103	as control	0	0
90	0	gross damage + leakage	0	0
200	0	coagulation of cytoplasm + surface protuberances,	6	14
500	0	no leakage	56	94

(*a*) Hugo & Longworth, 1964a.
(*b*) Hugo & Longworth, 1965.

substrate through the damaged cytoplasmic membrane. The inhibition of reduction of TTB in the presence of concentrations of chlorhexidine which cause gross damage to the cells and coagulate cytoplasm suggests that disruption of the cytoplasmic membrane and cytoplasmic coagulation inhibit the dehydrogenase activity of the organism. In view of the reaction of chlorhexidine with bacterial proteins it appears unlikely that the mode of action of the compound involves specific inhibition of a particular enzyme system.

It is proposed that the primary action of chlorhexidine consists of an adsorption of the drug onto a site on the surface of the cell. The adsorption is followed by a disorganization of the permeability barriers of the cell. The manifestations of the disruptive reaction depend on the concentration of chlorhexidine present. Low concentrations permit the

leakage of cytoplasmic constituents. Higher concentrations of chlorhexidine, which are used for antiseptic purposes and are more rapidly bactericidal, coagulate cytoplasmic constituents. It cannot be maintained that death is caused by leakage at all concentrations of the drug. An assessment of the relative bactericidal effects of membrane disruption and coagulation of cytoplasm must await the development of viable counting techniques which include a process to inactivate adsorbed drug and prevent agglutination of bacterial suspensions.

Helms & Weinberg (1962) studied the effect of a biguanide N^1,N^5-*d*-(3,4-dichlorobenzyl)biguanide (AM-1) on *Staphylococcus aureus* and their findings are not without interest in the light of our findings with chlorhexidine, a bis biguanidide.

Hugo & Longworth found that the stimulation of dehydrogenase activity occurred at a very low concentration of chlorhexidine (5 μg/ml). Apart from a slight leakage of cell constituents and an interfacial (cyclohexane/water) depression of about 2 dynes/cm, other effects at this concentration were negligible and the mean single survivor time was in excess of 200 hr. On the other hand, Helms & Weinberg found, with AM-1, optimal stimulation of dehydrogenase activity at 15 μg/ml which they declare to be precisely at the bactericidal concentration of the drug. However bactericidal activity was estimated by means of plate counts and an inactivator does not appear to have been used. Their high bactericidal activity might be due to a carry over of bactericide which continues to exert bacteriostatic or bactericidal effect during the incubation period necessary for plate counts. The authors show that lecithin reduces the bacteriolytic activity of AM-1 and it would have been interesting to see the result if lecithin had been used in conjunction with the viable count.

References

Giles, C. H. & McKay, R. B. (1965). *J. Bact.*, **89**, 390–397.
Helms, V. & Weinberg, E. D. (1962). *Antimicrobial Agents and Chemotherapy*, 241–249.
Holbrook, A. (1958). *J. Pharm. Pharmac.*, **10**, 370–374.
Hugo, W. B. (1954). *J. appl. Bact.*, **17**, 31–37.
Hugo, W. B. & Longworth, A. R. (1964a). *J. Pharm. Pharmac.*, **16**, 655–662.
Hugo, W. B. & Longworth, A. R. (1964b). *Ibid.*, **16**, 751–758.
Hugo, W. B. & Longworth, A. R. (1965). *Ibid.*, **17**, 28–32.
Kabat, E. A. & Mayer, M. M. (1961). *Experimental Immunochemistry*, 2nd edn, p. 559, Springfield: Thomas.
Longworth, A. R. (1965). Ph.D. Thesis, University of Nottingham.
Mejbaum, W. (1939). *Hoppe-Seyler's Z. physiol. Chem.*, **258**, 117–120.
McQuillen, K. (1952). Ph.D. Thesis, University of Cambridge.
Mickle, H. (1948). *Jl R. microsc. Soc.*, **68**, 10–12.
Moyer, L. S. (1936). *J. Bact.*, **31**, 531–546.
Rye, R. M. & Wiseman, D. (1964). *J. Pharm. Pharmac.*, **16**, 516–521.
Rye, R. M. & Wiseman, D. (1965). *Ibid.*, **17**, 295–301.
Salton, M. R. J. & Horne, R. W. (1951). *Biochim. biophys. Acta*, **7**, 177–197.
Strominger, J. L. (1957). *J. biol. Chem.*, **224**, 509–523.

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Simple methods for the detection of tropinesterase activity in rabbits*

R. M. QUINTON

In experiments in which rabbits are to be given atropine or certain atropine-like agents, it is important to select animals whose plasma is deficient in the enzyme tropinesterase (atropinesterase). Three simple procedures for assessing the presence of this enzyme in rabbits *in vivo* have been examined. It is concluded that a different procedure is needed for albino rabbits than for animals with dark irides. In this way, 95–98% correct assessments of the presence or absence of tropinesterase have been made by simple procedures within 1 hr. Fully reliable assessments could only be made by *in vitro* methods, two of which (one quantitative and the other qualitative) are described. The latter enabled a reliable all-or-none assessment of tropinesterase activity to be made with only 0·02 ml of plasma within 1–2 hr.

FLEISCHMANN (1910) and Metzner (1912) were the first to attribute the known variability in the sensitivity to atropine of rabbits in different laboratories, to the ability of the blood of certain individuals to hydrolyse atropine rapidly. At that time, rabbits whose blood possessed this ability appear to have had a geographical distribution. Thus Cyon (1898) and Fleischmann (1910) in Berne, Simon (1913) in Parma, Metzner (1912) in Basle, and Tardieu & Roussin in Paris (1867) reported a high sensitivity to the pharmacological actions of atropine in the rabbits they used, whereas most workers in Germany and Austria found rabbits noticeably insensitive to the alkaloid (e.g. Fröhlich (1874) and Rossbach (1875) at Wurzburg, Harnack (1874, 1898) at Strassburg and Halle, Furth & Schwarz (1908) in Vienna, and Gnauck (1881) in Berlin). Fleischmann and Metzner showed that the blood of the atropine-insensitive rabbits at Berlin, St. Ludwig and Leipzig possessed the power of inactivating atropine *in vitro*, whereas the more sensitive animals in Berne and Basle did not.

Since then mixing of regional rabbit strains has presumably taken place. By 1924 Rothlin reported that rabbits in Basle (where Fleischmann had found earlier only atropine-sensitive animals) showed a hundredfold variation in sensitivity to vagal block by atropine. More recently, Levy (1945) in France, Ambache & Lippold (1949), Hobbiger & Lessin (1955) and Brown & Quinton (1957) in England, Werner & Würker (1959) and Lendle & Paul (1964) in Germany, have all found a wide variation in sensitivity to atropine amongst the rabbits they used, and have shown this to run in parallel with the presence or absence of the enzyme tropinesterase (atropinesterase) in each animal's blood.

Since the presence of the enzyme in a rabbit's blood is an inheritable characteristic, probably of a sub-dominant nature (Sawin & Glick, 1943), the incidence of rabbits possessing this enzyme varies widely between stock in different laboratories or obtained from different breeders. In

From The Pharmacology Department, Pfizer Ltd., Sandwich, Kent, England.

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the experience of the present author no British strain or breed has been found to be completely free of animals possessing tropinesterase in their blood.

The enzyme is not specific for atropine and (—)-hyoscyamine, but also hydrolyses hyoscine, homatropine, benactyzine and many other amino-alcohol esters of tropic, benzilic, diphenylacetic and related acids (Werner, 1961; Herz, 1963; Quinton, unpublished).

It is obviously advisable therefore that, where rabbits are to be used in any pharmacological experiment involving atropine or a structurally similar ester, only those whose blood lacks tropinesterase should be chosen. This paper reports results obtained with various published methods for the ready detection of tropinesterase activity in rabbits and describes new procedures.

Experimental

In vivo METHODS

Three different procedures were used to assess the degree of pupil dilatation produced by a low dose of atropine or hyoscyamine. In all instances, the pupils were illuminated by a 60 W bulb of an Anglepoise lamp, partly masked to give a light aperture of 4 cm and placed 30 cm from the eye. The diameters of both pupils were measured, with a transparent metric rule held close to the eye, immediately before and at a stated time after treatment. A change in pupil diameter of 1 mm and more was considered significant. Measurement of pupil size was performed by assistants who did not know what changes to expect.

(i) One drop containing 1 μg of hyoscyamine sulphate was placed in the conjunctival sac of one eye of a rabbit. The degree of pupil dilatation was measured 60 min later. Under these conditions rabbits lacking tropinesterase have been reported to give a positive mydriatic response, whereas those in which the enzyme is present do not (Werner & Würker, 1959).

(ii) Rabbits were injected intravenously with either 0·1 or 1·0 mg/kg atropine sulphate. The diameters of both pupils were measured again 15 or 60–75 min respectively after injection. Under both these conditions the pupils of rabbits possessing tropinesterase have been reported to regain the light reflex (i.e. to contract in bright light) whereas in those lacking the enzyme the reflex remains blocked (i.e. the pupils stay dilated) (Ambache, Kavanagh & Shapiro, 1964).

(iii) The conjunctival sac of one eye of a rabbit was flooded with 2–3 drops of a solution containing 2·5 mg/ml of physostigmine sulphate and 0·3 mg/ml of atropine sulphate in 0·9% saline; the estimated amounts instilled were about 300 and 36 μg respectively. Change in pupil diameter was measured 45 min later.

In vitro METHODS

Plasma was separated by centrifugation from blood withdrawn from an ear vein and heparinized. It was stored at -17° when not in use;

under these conditions no significant loss in tropinesterase activity was noted within 3 months.

Incubation of plasma with atropine, followed by bioassay of residual atropine. To 0·25 ml of plasma in a 10 ml measuring cylinder were added 8·75 ml of a phosphate buffer pH 7·4 (20 ml of 5% w/v NaH_2PO_4 plus 20 ml of 5% w/v $Na_2HPO_4.2H_2O$ in 1 litre 0·9% w/v NaCl, brought to pH 7·4 with 20% w/v NaOH). The cylinder was placed in a water-bath kept at 37·7 ± 0·2°. After 5 min, 1 ml of a 6 mg/ml solution of atropine sulphate was added and the cylinder inverted twice to mix the contents. The enzymatic action was stopped either 30 or 60 min later by adding three drops of N hydrochloric acid; two drops of Universal Indicator solution (B.D.H.) were added at the same time to check that the pH of the solution was below 3. Incubated solutions were always assayed within 30 hr, being stored at −17° overnight.

Residual atropine in the incubated solutions was assayed by its mydriatic action in mice (Pulewka, 1932). Details of the method have been described previously (Quinton, 1963). Male albino mice, 25–40 g, were injected subcutaneously with 0·1 ml solution/10 g and the right pupil diameter measured again 40 min after injection. The response was taken as the arithmetic difference between the initial and the final pupil diameter. The potency of a solution was determined by reference to a logarithmic dose-response line derived from two dilutions of a non-incubated solution of atropine sulphate prepared at the same time as the incubated solutions and in exactly the same manner, except that buffer solution replaced the 0·25 ml of plasma. Generally, dilutions of 1–60 and 1–150 (corresponding to doses of atropine sulphate of 1 and 0·4 μg/10 g) gave suitable mydriatic responses of about 75% and 25% respectively of the maximum possible; 10–15 mice were used for each solution. The significance of marginal differences in potency between any test and the standard solution was checked by Student's "*t*" test applied to the mydriatic responses obtained from identical dilutions of the two solutions. Tropinesterase activity in each plasma sample was expressed as μg atropine base/ml of incubated solution hydrolysed in 1 hr under the conditions specified here.

No significant non-enzymatic inactivation of atropine could be demonstrated after incubation for 3 hr.

Agar plate method. The method was derived from that of Sandi (1962) who devised it to detect organophosphate anticholinesterase insecticide residues in plant material. Atropine sulphate, 5 mg/ml, was dissolved in a suspension of 1·5% w/v agar in a weak phosphate buffer (4 ml 0·1 M KH_2PO_4 plus 40 ml 0·1 M Na_2HPO_4, to 1 litre with 0·9% saline, and brought to pH 8·0 with 0·1 N NaOH). The pH of this solution after adding the agar and atropine sulphate was about 7·6. Sufficient solution of bromothymol blue was added to the solution after boiling to give a final concentration of about 0·02% w/v, and the mixture poured into petri dishes.

In earlier work plasma was added to circular holes cut in the agar, but more rapid results requiring smaller amounts of plasma were obtained

if small filter paper discs (Whatman No. 42) were used instead. The discs were about 6 mm diameter, cut by an office stationery hole-punch and placed on the surface of the agar. For a petri dish of 8·5 cm diameter, 12–15 ml of agar solution gave a suitable depth of agar of 2–3 mm. Only 0·020 ml of plasma (applied as three drops from a syringe fitted with a number 18 hypodermic needle) were needed; the plasma was dropped on to three discs of filter paper placed one on top of the other.

For rapid results, the agar plates were incubated at 38 or 45°. It was found that plastic petri dishes could be floated safely in water-baths at these temperatures, with lids placed upside-down on top to protect the agar from any splashes. Under these conditions colour changes were visible within 30 min and quite distinct by 60–120 min. Around filter paper discs to which tropinesterase-containing plasma had been added, enzymatic hydrolysis of the atropine caused a fall in pH with a corresponding colour change of the indicator from blue to yellow. In the case of plasma not containing tropinesterase, the colour remained blue or green, but never turned to yellow.

Results

In vivo METHODS

The changes in pupil size caused by the three different treatments with atropine or hyoscyamine (see "Methods") were assessed in about 150 mature rabbits of seven breeds (and a number of crosses).

Results, expressed as the percentage of animals with or without tropinesterase which responded in the manner predicted by Werner & Würker or Ambache & others, are represented in Fig. 1. Totals are shown, plus separate histograms for albino and non-albino animals. The tropinesterase activity of the plasma of each rabbit was determined by incubation with atropine *in vitro*, and subsequent bioassay of the residual atropine.

After instillation of 1 μg hyoscyamine sulphate in the eye, 18 out of 24 rabbits whose blood lacked tropinesterase activity showed a significant mydriatic response. Of these, seven were albinos, all of which responded. Of the rabbits possessing tropinesterase, 23 out of 25 (nine out of 10 albinos) failed to show a significant pupil dilatation. In all, 84% of all rabbits (94% of albinos) gave responses in this test of the nature predicted (Werner & Würker, 1959).

In the second procedure (Ambache & others, 1964), the pupils of all 16 rabbits (including eight albinos) lacking tropinesterase showed significant dilatation when examined 15 min after an intravenous injection of 0·1 mg/kg of atropine sulphate. Of the animals in which the enzyme was present, 16 out of 25 (three out of eight albinos) failed to show dilatation. The overall proportion responding as expected was 78% (69% for albinos only).

After the alternative treatment (1·0 mg/kg atropine sulphate 60–75 min previously) only 13 out of 22 rabbits (three out of five albinos) gave mydriatic responses as expected.

Analysis of the failures of rabbits to respond as expected in these two tests suggested that the main source of error lay in the marked difference in sensitivity of albino and non-albino rabbits to the mydriatic action of atropine. Thus the pupils of five out of eight albinos with tropinesterase dilated significantly to 0·1 mg/kg of atropine, whereas six out of 24 non-albino animals lacking tropinesterase failed to show a significant mydriatic

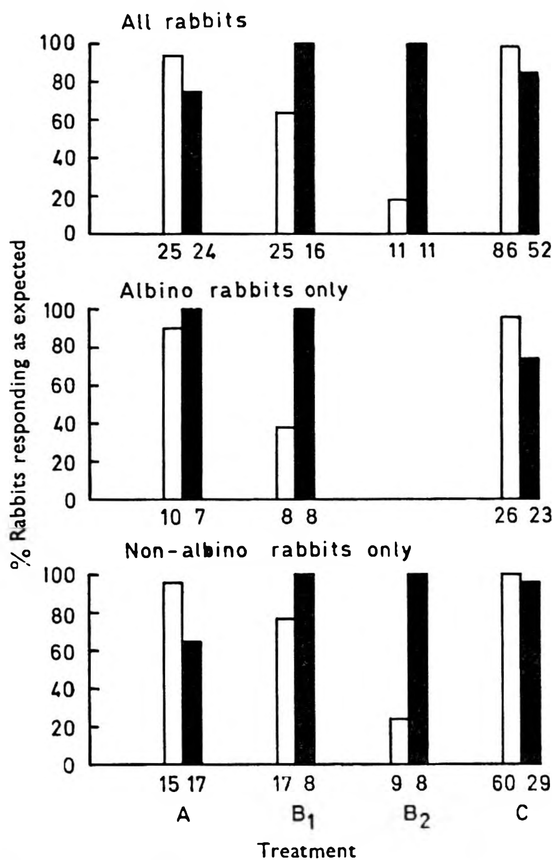

FIG. 1. Percentage of rabbits, with (open columns) or without (solid columns) significant plasma tropinesterase activity, responding by changes in pupil size as predicted (see text) to various treatments with atropine or hyoscyamine sulphate. Treatment: A, 1 μg hyoscyamine sulphate in eye; B1 or B2, 0·1 or 1·0 mg/kg atropine sulphate i.v.; C, 36 μg atropine sulphate + 300 μg physostigmine sulphate in eye. Figures indicate numbers of animals from which each percentage value is derived.

response to 1 μg hyoscyamine in the eye. These differences were shown also in the resting state since, under the lighting conditions used in this work, the pupils of albinos measured 4–5 mm before treatment, whereas those with dark irides were 5½–7 mm in diameter.

It was thought possible that any mydriatic action of atropine might be more readily discernible in the eyes of non-albino rabbits if the pupils

were first contracted by physostigmine. In the third type of test, therefore, physostigmine was instilled in the eye at the same time as atropine in a suitable concentration to cause mutual antagonism. Thus, in rabbits lacking tropinesterase, it was expected that the pupil sizes would not be altered significantly by this treatment, whereas in animals possessing the enzyme sufficient atropine would be destroyed for the physostigmine to produce miosis.

In the former group, the pupils of 44 out of 52 animals showed no change in size; of these 27 out of 29 were non-albino. In the case of rabbits with tropinesterase, 85 out of 86 displayed significant miosis. The animal which failed to respond in this way was an albino.

Thus, in the case of the *non-albino* rabbits, the method gave responses as expected in 87 animals out of 89, or 98%.

It was noted that even though the miotic action of physostigmine was presumably indirect, via inhibition of cholinesterase, its effects were noticeable within 10–20 min, before any atropine antagonism was seen. By 45 min after instillation, however, both drugs were exerting their effects and this was the time chosen for observation of any change in pupil size.

In vitro METHODS

Incubation of plasma with atropine, followed by bioassay of residual atropine. This method has been used routinely to determine tropinesterase activity in the plasma of over 300 rabbits during the past 8 years.

The limits of error of an estimate of the potency of an incubated solution were smallest when hydrolysis had been allowed to continue until its atropine content* was low, since it needed to be diluted less before assay. On the other hand, hydrolysis was not allowed to proceed beyond 75% of the atropine present, to ensure a nearly maximum rate of esterase activity. Except where an exact value for a particularly low hydrolytic activity was required, it was usually adequate to incubate solutions of atropine with plasma for either 30 or 60 min (depending on the tropinesterase activity). In these cases, significant tropinesterase activity could be detected down to values of about 90 μg atropine hydrolysed/ml/hr.

In rabbits possessing significant plasma tropinesterase activity, calculated values ranged from 78 to 720 μg atropine hydrolysed/ml solution/hr.

Agar plate method. This test has given consistently reliable results with rabbit plasma samples, in qualitative agreement with the assessment of tropinesterase activity made by incubation with atropine *in vitro* and subsequent bioassay of the residual atropine. Although the colour changes induced by tropinesterase-containing plasmas might be quantified by some colorimetric instrument, this has not been done since it was considered that the method could never attain the accuracy of the incubation/bioassay method described above.

* The presence of (+)-hyoscyamine in the incubated and assayed solutions was ignored, since it is hydrolysed only very slowly by tropinesterase (Werner & Brehmer, 1959) and has only 1/40–1/100 of the potency of the (−)-isomer in the mouse mydriatic test.

Degrees of colour change could however be observed by the naked eye and correlated with the tropinesterase activity of the plasma samples used. The time-courses of the colour changes produced by three plasma samples of differing tropinesterase activity and two samples lacking any activity are shown in Fig. 2, for three different temperatures of incubation.

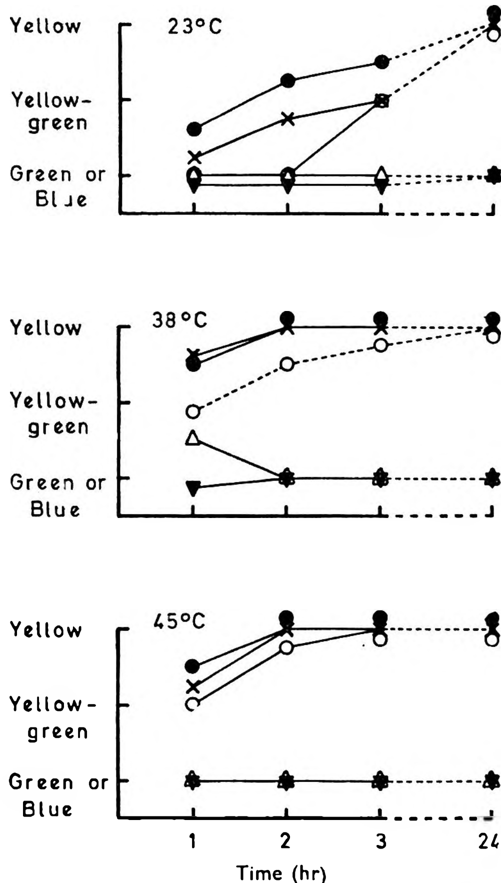

FIG. 2. Time-course of the colour changes caused in agar plates containing atropine sulphate (5 mg/ml) and bromothymol blue, by plasma (0·02 ml) of rabbits with various tropinesterase activities. ●, plasma tropinesterase activity of 678 μg atropine hydrolysed/ml solution/hr; ×, 272 μg/ml/hr; ○, 110 μg/ml/hr; △, no significant activity; ▼, no significant activity. Each point represents the mean of 2 or 3 observations.

With the plasma possessing the weakest activity, a colour change to yellow-green could be observed after 60 min at 38° or 45°, but was more convincing after 120 min. Plasma samples lacking tropinesterase occasionally produced a slight initial colour change towards yellow-green, but this was never seen after incubation for 120 min or more.

The parameters described for this method were chosen to give clear-cut

colorimetric responses within 60–120 min with the use of as little plasma and atropine as possible. Thus 0·02 ml of plasma and 5 mg/ml of atropine sulphate gave good responses whereas the colour changes by 0·007 ml or 2 mg/ml respectively were less convincing. The composition of the buffer solution did not appear critical providing it had only weak buffering capacity within the pH range 6·0–7·6. (Preliminary experiments in which a standard Sorensen phosphate buffer of pH 7·6 was used gave disappointing results before it was realised that the buffer was effectively resisting the pH change caused by hydrolysis of the atropine). Temperatures of 38 or 45° were found best for rapid colour changes, although perfectly adequate colour changes could be obtained even in plates kept at 4°, for 18–24 hr.

Enzymatic activity apparently fell off markedly below about pH 6, since, if bromocresol purple was used, it was changed to a pure yellow colour (pH 5·2) only after 12–24 hr at 38°. The small pH range suitable, about 6·0–7·4, therefore restricted the choice of indicators. Bromothymol blue, changing over exactly this range, gave clear-cut colour changes.

Attempts to use this method for whole blood were not fully successful, although distinctive colour-changes could be obtained from 0·015–0·03 ml blood. Since however the blood samples needed much longer incubation than did plasma, and colour changes were not so clear-cut, plasma was preferred.

Discussion

The object of this work was to examine various simple methods whereby tropinesterase activity could be assessed in rabbits, both in the whole animal and in the blood or plasma *in vitro*. Techniques requiring much equipment or expertise have not been considered (such as the Warburg manometric method, as used by many workers in this field—e.g. Glick, 1940). Particular attention has been paid to techniques which would permit a speedy all-or-none assessment of tropinesterase presence in the blood of a rabbit.

In the intact animal, none of the three methods described gave a completely reliable indication of the presence or absence of tropinesterase activity. This was probably due to marked differences in sensitivity to the mydriatic action of atropine or hyoscyamine in albino and non-albino rabbits. Thus, some albino animals possessing tropinesterase gave significant mydriatic responses to low doses of atropine or hyoscyamine administered topically or by intravenous injection. Conversely, the pupils of some rabbits with dark irides failed to show a significant dilatation to these agents even when no tropinesterase was present.

For these reasons it is recommended that albino and non-albino rabbits should be treated differently. Instillation of 1 μg of hyoscyamine sulphate in one eye of an albino rabbit has been shown, in 94% of animals, to cause significant mydriasis if the animal lacks tropinesterase, but no change in pupil size if its blood contains the enzyme. For rabbits with dark irides, instillation in one eye of a mixture of physostigmine sulphate and atropine

sulphate (about 300 μg and 36 μg respectively) has been shown, in 98% of animals, to produce significant pupil contraction in animals with tropinesterase but no significant change in pupil size in those lacking the enzyme. The two exceptions in the 89 non-albino rabbits tested by this procedure were tropinesterase-free but their pupils did contract significantly; in other words they reacted as if they possessed tropinesterase. All the rabbits whose pupils did *not* contract significantly were tropinesterase-free. The test therefore appears to offer a reliable means of selecting tropinesterase-*free* rabbits.

Under the standard lighting conditions used it was noted that, in their resting state, the pupils of albino rabbits were significantly more contracted than those of rabbits with pigmented irides. Possibly if the strength of lighting had been adjusted for each rabbit to give a constant initial pupil size, as was done by Herz (1963) when he used the procedure of Werner & Würker (1959), these differences in sensitivity to the mydriatic action of atropine or hyoscyamine might not have been observed. Lighting conditions were kept constant here, however, since it was felt that adjustment of lighting for each individual rabbit introduced an undesirable complication into what were intended to be simple procedures. Furthermore, experience had shown that the use of stronger illumination than that described here induced some rabbits to keep their eyes firmly closed.

Two methods, one quantitative and the other qualitative, have been described for the assessment of tropinesterase activity in rabbit plasma *in vitro*. The quantitative method, involving incubation of plasma with atropine followed by bioassay of the residual atropine, has been used routinely for the definitive assessment of tropinesterase activity in the rabbits used for the tests *in vivo*. The qualitative test makes use of the colour change induced in a pH indicator by the acid produced on hydrolysis of atropine by tropinesterase, in an agar plate. It needs very small quantities of plasma, 0·02 ml giving an adequate colour change even with plasma samples possessing feeble tropinesterase activity. It is simple and quick, a reliable qualitative assessment of enzyme presence being possible within 120 min.

If a rabbit of unknown tropinesterase activity should at any time be required to be used immediately for an experiment in which atropine or a closely-related ester is to be given, it is probably preferable to use a quaternary ammonium salt such as atropine methyl nitrate, which is not hydrolysed by tropinesterase (Dirner, 1937). Its ganglion-blocking activity (Bainbridge & Brown, 1960) and failure to pass the blood-brain barrier may, however, be disadvantageous. In such circumstances, (−)-tropine α-methyl tropate (Kramer, Maffii & Quinton, to be published) or oxyphencyclimine (Finkelstein, P'an, Niesler, Johnson & Schneider, 1959) could be used; these agents are not significantly hydrolysed by rabbit tropinesterase (Quinton, to be published).

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References

Ambache, N., Kavanagh, L. & Shapiro, D. W. (1964). *J. Physiol., Lond.*, **171**, 1P–2P.
Ambache, N. & Lippold, O. C. J. (1949). *Ibid.*, **108**, 186–196.
Bainbridge, J. G. & Brown, D. M. (1960). *Br. J. Pharmac. Chemother.*, **15**, 147–151.
Brown, D. M. & Quinton, R. M. (1957). *Ibid.*, **12**, 53–60.
Cyon, E. von (1898). *Pflügers Arch. ges. Physiol.*, **73**, 43–70.
Dirner, Z. (1937). *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **186**, 409–427.
Finkelstein, M., P'an, S. Y., Niesler, V. N., Johnson, C. A. & Schneider, J. A. (1959). *J. Pharmac. exp. Ther.*, **125**, 330–338.
Fleischmann, P. (1910). *Berl. klin. Wschr.*, **48**. 135–136.
Fröhlich, C. (1874). *Verh. phys.-med. Ges. Würzb.*, **6**, 190–236.
Furth, O. von & Schwarz, K. (1908). *Pflügers Arch. ges. Physiol.*, **124**, 113–156.
Glick, D. (1940). *J. biol. Chem.*, **134**, 617–625.
Gnauck (1881). *Arch. Anat. Physiol.* (physiol. Abt.), 466P–471P.
Harnack, E. (1874). *Arch. exp. Path. Pharmak.*, **2**, 307–333.
Harnack, E. (1898). *Pflügers Arch. ges. Physiol.*, **70**, 126–280.
Herz, A. (1963). *Archs int. Pharmacodyn. Thér.*, **141**, 595–604.
Hobbiger, F. & Lessin, A. W. (1955). *J. Physiol., Lond.*, **128**, 71P.
Lendle, L. & Paul, H. A. (1964). *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **249**, 295–305.
Levy, Jeanne (1945). *Bull. Soc. Chim. biol.*, **27**, 584–596.
Metzner, R. (1912). *Arch. exp. Path. Pharmak.*, **68**, 110–159.
Pulewka, P. (1932). *Nauyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **168**. 307–318.
Quinton, R. M. (1963). *J. Pharm. Pharmac.*, **15**, 239–250.
Rossbach, M. J. (1875). *Pflügers Arch. ges. Physiol.*, **10**, 383–484.
Rothlin, E (1924). *Ibid.*, **206**, 614–628.
Sandi, E. (1962). *Die Nahrung, Berl.*, **6**, 57–67.
Sawin, P. & Glick, D. (1943). *Proc. Natn Acad. Sci. U.S.A.*, **29**, 55–59.
Simon, I. (1913). *Archo Farmac. sper.*, **15**, 254–267, through *Chem. Zentbl.*, **2**, 525.
Tardieu & Roussin (1867). *Etude medico-légale et clinique sur l'empoisonnement*, Paris, quoted by Rabuteau, A. (1873). *Un. méd.*, 3*ème* Sér., **16**, 1006–1010.
Werner, G. (1961). *Planta med.*, **9**, 293–316.
Werner, G. & Brehmer, G. (1959). *Naturwissenschaften*, **46**, 600–601.
Werner, G. & Würker, R. (1959). *Ibid.*, **46**, 627.

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A basic model for the evaluation and prediction of preservative action

EDWARD R. GARRETT

A basic integrated model for the quantification of preservative action must consider the availability or thermodynamic activity of the biologically effective concentration, μ, of the preservative in the aqueous phase. A derived expression for the total concentration, P_T, of preservative (of dissociation constant K_a) degrading by an apparent first order rate constant, k′, in an oil/water emulsion (of q volume ratio and k intrinsic partition coefficient of undissociated preservative) needed to maintain a minimum inhibitory concentration μ of free undissociated acid in the aqueous phase at any hydrogen ion concentration [H⁺] and for any known binding or complexing phenomena (where there are n sites on binding macromolecule M_i and the intrinsic dissociation constant is k_i) is $P_T = \mu\,(f_1 \times f_2 \times f_3)$. The binding enhancement factor of μ is

$$f_1 = 1 + \sum_{i=1}^{n} n_i\,[M_i]/[K_i + \mu\,(1 + K_a/[H^+] + kq)$$

and in many practical instances when $\mu \ll K_i$ it reduces to

$$f_1 = 1 + \sum_{i=1}^{n} n_i\,[M_i]/K_i$$

The oil/water partition and ionization enhancement factor is $f_2 = 1 + K_a/[H^+] + kq$ where kq vanishes in the absence of oil. The instability enhancement factor is $f_3 = e^{k't}$. The ultracentrifuge can be used to define operationally the parameters in macromolecular binding and the apparent partition in dispersions, emulsions and solutions. The premises for the use of preservative combinations are critically evaluated and kinetic methods to determine proper choices of response to characterise combined preservative action are recommended.

NUMEROUS authors (Wyss, 1948; Reddish, 1957; Sykes, 1958; Bennett, 1959; Tice & Barr, 1959; Cook, 1960; Jacobs, 1960; de Navarre, 1962; Bean, Heman-Ackah & Thomas, 1965) have considered the various factors in the choice of preservatives in food, pharmaceuticals and cosmetic preparations. But there has been little attempt to systematically quantify all the pertinent factors so that they can be included in one basic integrated model. The chemical preservatives I wish to consider are those substances inhibiting or destroying micro-organisms which may contaminate or grow in pharmaceuticals or food preparations (Tice & Barr, 1959; de Navarre, 1962).

A BASIC MODEL FOR THE QUANTIFICATION OF PRESERVATIVE ACTION

A basic model for preservative action must consider the availability or thermodynamic activity of the biologically effective concentration of the preservative in the aqueous phase (Rahn & Conn, 1944; Garrett & Woods, 1953; Anton, 1960, 1961). With organic acids, the undissociated (Rahn & Conn, 1944; Garrett & Woods, 1953) and unbound (Allawala & Riegelman, 1953; Patel & Kostenbauder, 1958; Miyawaki, Patel & Kostenbauder, 1959; Pisano & Kostenbauder, 1959; Anton, 1960, 1961) fraction is the effective species.

From the College of Pharmacy, University of Florida, Gainesville, Florida.

The basic premises of such an approach are that (i) inhibition or kill of a species of micro-organisms may be accomplished by a finite concentration of biologically active material, i.e. a minimum inhibitory concentration, μ, and (ii) the vehicle for growth of the micro-organism is aqueous and the biological activity must be exercised in the aqueous phase. The problem reduces itself for a single chemical preservative to one of thermodynamic availability equal to, or in excess of, the minimum inhibitory concentration, μ, of the active preservative species.

QUANTIFICATION OF PRESERVATIVE BINDING TO MACROMOLECULES AND OF COMPLEX FORMATION

The phenomena of binding to surfactants, proteins or other macromolecules can effectively reduce preservative (Allawala & Riegelman, 1953; Patel & Kostenbauder, 1958; Miyawaki & others, 1959; Pisano & Kostenbauder, 1959) or anti-bacterial (Anton, 1960, 1961) activity against a variety of micro-organisms. The quantification of such binding may be considered as being similar to those expressions established for the protein binding of drugs (Klotz, 1946, 1953; Goldstein, 1949).

A classical model is that of a macromolecule, M, having n independent binding sites with an intrinsic dissociation constant, K, for the binding of a molecule of preservative, P, to one of these sites. If $[M_n]$ is the concentration of sites in equivalents/litre then $[M_n] = n\,[M]$ initially.

$$P + M_n \overset{K}{\rightleftharpoons} PM_n \qquad (1)$$

The concentration of bound preservative, $[PM_n]$ per concentration of the total macromolecule, $[M]$, is

$$r = [PM_n]/[M] = n\mu'/(K + \mu') \qquad (2)$$

where μ' is the concentration of unbound preservative.

The values of n and K can be obtained from dialysis, ultrafiltration or ultracentrifugal analysis by classical procedures. The equation 2 can be rearranged to

$$r/\mu' = n/K - (1/K)r \qquad (3)$$

The moles of preservative bound per mole of macromolecule, i.e. r, when divided by the concentration of free preservative, i.e. μ', give a straight line when plotted against r with a negative slope of $1/K$ and an intercept of n/K (Fig. 1). Thus n and K can be calculated. By equilibrium dialysis, the concentration of preservative external to the dialysis bag permits the estimation of μ.

If μ' is the concentration of unbound preservative needed, then

$$P_T = \mu' + [PM_n] \qquad (4)$$

where P_T is the total concentration of preservative needed to maintain an unbound concentration, μ', where $[PM_n]$ is the apparent concentration of bound preservative. It follows from equations 2 and 4 that

$$P_T = \mu' \{1 + n[M]/(K + \mu')\} = \mu' f_1 \qquad (5a)$$

or

$$P_T = \mu' \{1 + n[M]/K\} = \mu' f_1 \qquad (5b)$$

where $K \gg \mu'$ and f_1 may be considered as the concentration enhancement factor to correct for macromolecular binding of preservative in the aqueous phase.

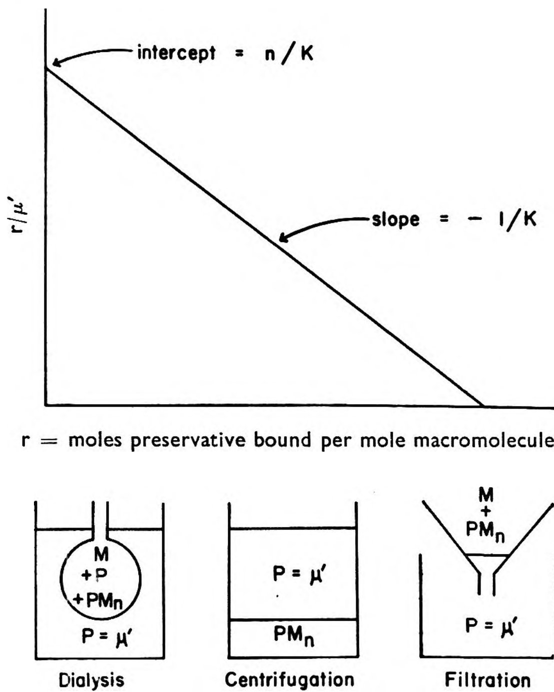

FIG. 1. Typical plot of data to obtain parameters for binding of preservatives to macromolecules, M. The concentrations of unbound preservative $P = \mu'$, are assayed after equilibrium dialysis, centrifugation, or filtration. From these data the total volume of the solution and the total amounts of macromolecule and preservative, $P + PM_n$, the moles of preservative P bound per mole of macromolecule, $r = [PM_n]/[M]$ can be calculated. The equation of the plot is $r/\mu' = n/K - (1/K)r$. r/μ' = moles preservative bound per mole macromolecule/concentration of unbound preservative.

If there are several types of macromolecules of concentrations $[M_1]$, $[M_2]$, $[M_3]$ . . . $[M_m]$ with numbers of binding sites per molecule n_1, n_2, n_3, . . . n_m and with intrinsic dissociation constants, K_1, K_2, K_3 . . . K_m; it follows by a reasoning similar to that for equation 5 that

$$P_T = \mu' \{1 + n_1 [M_1]/(K_1 + \mu') + n_2 [M_2]/(K_2 + \mu')\} + \ldots + n_m[M_m]/(K_m + \mu')\}$$

$$= \mu' \{1 + \sum_{i=1}^{m} n_i [M_i] /(K_i + \mu')\} = \mu' f_1 \qquad (6a)$$

or $$P_T = \mu' \{1 + \sum_{i=1}^{m} n_i [M_i] /K_i\} = \mu' f_1 \qquad (6b)$$

for the conditions where the macromolecular binding of the preservative is weak or the concentration of preservative is low.

The formation of biologically inactive complexes of preservatives with other compounds in solution is analogous to the development given for macromolecular binding. For a 1:1 stoichiometric complex, $n = 1$, and [M] is the concentration of the complexing agent. It follows that the equations 5 and 6 may represent the total concentration of preservative, P_T, necessary to maintain an effective concentration, μ', for the combined cases of macromolecular binding and molecular complexing.

However, dialysis and ultracentrifugation are not applicable techniques to determine the stoichiometry and the dissociation constants of such molecular complexes. Studies on the interaction of the complexing species can best be conducted by spectrophotometric (Job, 1928; Vosburgh & Cooper, 1941), potentiometric (Bjerrum, 1941; Calvin & Melchior, 1948; Martell & Frost, 1950), partition (Higuchi & Zuck, 1953; Guttman & Higuchi, 1957) and solubility analysis (Higuchi & Lach, 1954a, b). If $n > 1$, the f_1 function of equations 5 and 6 may be modified in accordance with the cited references when multiple K values are needed for multiple complexes.

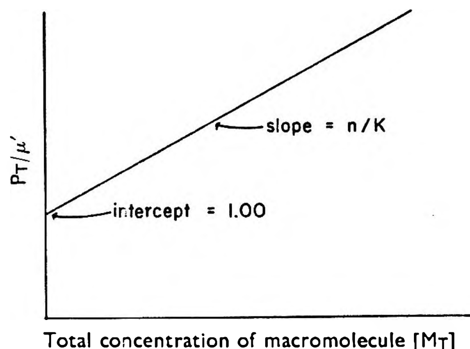

FIG. 2. Binding of preservatives to macromolecular surfactants (after Patel & Kostenbauder, 1958; Pisano & Kostenbauder, 1959). The plot is consistent with $P_T/\mu' = 1 + (n/K)[M_T]$ where n is the number of binding sites on the macromolecule and K is the apparent equilibrium constant. P_T/μ' = ratio of total preservative to amount unbound.

The linearity of the plot of the ratio, P_T/μ' (of the total preservative concentration, P_T, to the concentration of the unbound preservative, μ) against an increase in concentration, [M], of the macromolecule in accordance with a transformation of equation 5

$$P_T/\mu' = 1 + (n/K)\,[M] \qquad (7)$$

has been demonstrated practically for the parabens by Kostenbauder and associates (Allawala & Riegelman, 1953; Patel & Kostenbauder, 1958; Miyawaki & others, 1959; Pisano & Kostenbauder, 1959) as in Fig. 2. The intercept of such plots is the expected unity. It is then apparent that the simplified expression of equations 5b and 6b are good approximations of the binding enhancement factor, f_1.

Quantification of preservative activity as a function of pH. Many preservatives are distributed among ionic and nonionic species as a function of pH. Amongst the acid preservatives, the non-charged form is the active preservative species in many instances (Rahn & Conn, 1944; Garrett & Woods, 1953). The dissociation constant for an acid preservative HA may be defined in

$$[A^-] = K_a [HA]/[H^+] \quad .. \quad .. \quad .. \quad (8)$$

If [HA] is added to both sides of equation 8, it can be rearranged to give the fraction, f_{HA}, of the total preservative that is associated as a function of the dissociation constant and the hydrogen ion concentration

$$f_{HA} = [HA]/([HA] + [A^-]) = 1/(1 + K_a/[H^+]) \quad .. \quad .. \quad (9)$$

The effective minimum inhibitory concentration of the undissociated acid preservative that is unbound is μ and is related to the effective minimum inhibitory concentration μ for all ionic species by the expression

$$\mu = f_{HA}\mu' \quad .. \quad .. \quad .. \quad .. \quad (10)$$

It follows from equations 9 and 10 that

$$\mu' = \mu/f_{HA} = \mu\{1 + K_a/[H^+]\} = f_2'\mu \quad .. \quad .. \quad (11)$$

Similarly, if the preservative active species is a non protonated amine of dissociation constant for the protonated amine of K_a

$$\mu' = \mu/f_{RNH2} = \mu\{1 + [H^+]/K_a\} = f_2'\mu \quad .. \quad .. \quad (12)$$

Quantification of preservative activity in the continuous aqueous phase of oil/water emulsions. In the specific instance of preservative activity in an emulsion, the partition between the oil and water phases will diminish the effective preservative activity. An operational model for predictive purposes can be established on the premises that the phases are immiscible, that there is no dissociation in the organic phase, that concentration of the charged and uncharged species are approximately equal to their thermodynamic activities and that the undissociated acid molecules are distributed between the oil and water phases by the partition law

$$k = [HA]_{oil}/[HA]_w = [HA]_{oil}/\mu \quad .. \quad .. \quad .. \quad (13)$$

where the ratio of the concentrations of undissociated acid molecules in the oil phase to the water phase is a constant called the intrinsic distribution constant, k.

It has been shown (Garrett & Woods, 1953) that the fraction f_{HAaq} of the total preservative acid that is both undissociated and in the aqueous phase can be given by the expression

$$f_{HAaq} = 1/(1 + K_A/[H^+] + kq) \quad .. \quad .. \quad .. \quad (14)$$

where q is the volume ratio of oil to water phase.

The effective minimum inhibitory concentration of the undissociated acid preservative that is in the aqueous phase is μ and is related to the effective minimum inhibitory concentration μ' for all ionic species in the total oil/water emulsion by the expression

$$\mu = f_{HAaq}\mu' \quad .. \quad .. \quad .. \quad .. \quad (15)$$

It follows from equations 14 and 15 that

$$\mu' = \mu\{1 + K_A/[H^+] + kq\} = \mu f_2 \quad .. \quad .. \quad (16)$$

It is interesting to note that if no oil is present or if the material does not significantly partition, kq = O and equation 16 reduces to equation 10.

Equation 16 can be modified for the case where a non-protonated amine in the aqueous phase is the active preservative.

Integrated model for the quantification of preservative action. Combination of equations 6a and 16 produces an expression for the total concentration, P_T, of preservative in an oil/water emulsion, i.e. moles preservative per total volume, needed to maintain a minimum inhibitory concentration μ of free undissociated acid in the aqueous phase at any hydrogen ion concentration and for any known binding or complexing phenomena. The expression is very like the development of Krüger-Thiemer for the pharmacokinetic expression of drug dosage for chemotherapeutic effect in the body (Krüger-Thiemer, Diller, Dettli, Bünger & Seydel, 1964) in that $(P_T)_o = \mu$ (minimum inhibitory concentration of active form of preservative)

X f_1 (binding enhancement factor of μ)
X f_2 (oil/water partition and ionization enhancement factor)
X f_3 (instability enhancement factor) (17)

It is possible to consider that preservative instability is directly analogous to Krüger-Thiemer's "pharmacokinetic factor".

If a first order decomposition of rate constant, k', is assumed for the preservative, then

$$P_T = (P_T)_o e^{-k't} \quad .. \quad .. \quad .. \quad .. \quad (18)$$

where $(P_T)_o$ would be the total concentration of preservative at time t_o to maintain a minimum concentration of P_T for time, t, at a given temperature.

The complete expression for the initial concentration of a preservative $(P_T)_o$ necessary for the maintenance of a minimum inhibitory concentration of the biologically active unbound, undissociated species in the aqueous phase for a time, t, would be

$$(P_T)_o = \mu f_1 f_2 f_3 = \mu\{1 + \sum_{i=1}^{m} n_i [M_i]/[K_i + \mu (1 + K_a/[H^+] + kq)]\}\{1 + K_a/[H^+] + kq\}\{e^{k't}\} \quad .. \quad .. \quad .. \quad (19a)$$

From what has been stated previously, in many practical instances of macromolecular binding, $\mu \ll K_i$ and equation 19a can be reduced to

$$(P_T)_o = \mu\{1 + \sum_{i=1}^{m} n_i [M_i]/K_i\}\{1 + K_a/[H^+] + kq\}\{e^{k't}\} \quad .. \quad (19b)$$

If there is only one binding or complexing species and no oil for partition, kq = O and

$$(P_T)_o = \mu\{1 + n [M]/K\}\{1 + K_a/[H^+]\}\{e^{k't}\} \quad .. \quad .. \quad (19c)$$

In the special instance of a non-ionizable preservative that is stable, equations 19 reduce to equations 5 or 6 where $\mu' = \mu$.

An operational method for the experimental determination of the necessary parameters for the prediction of necessary preservative concentration in a complex system. The amount of unbound material in the aqueous phase of an oil/water emulsion with surfactant molecules could be determined by ultracentrifugation. The lighter oil droplets would readily tend to cream and clear the emulsion (Garrett, 1962) under centrifugal stress. Analysis of the total preservative concentration, P_{aq}, in the cleared volume after a very short period of ultracentrifugation would permit the evaluation of the total preservative bound and unbound to the equilibrium surfactant concentration in the aqueous phase, i.e.

$$P_{aq} = \mu'_{aq} + [PM_n] \quad .. \quad .. \quad .. \quad .. \quad (20)$$

where μ' and $[PM_n]$ are concentrations of unbound and bound preservative in the aqueous phase respectively. The equilibrium surfactant concentration in the sample of the aqueous phase can also be assayed. A typical procedure to obtain the concentration [M] of a macromolecule in this aqueous phase is given by MacCallister & Lisk (1951). It is possible to determine [M] as a function of the initial concentration $[M]_T$ of the macromolecule in the total preparation by repeating the ultracentrifugation and analysis as a function of variable $[M]_T$ values so that

$$[M] = f[M]_T \quad .. \quad .. \quad .. \quad .. \quad (21)$$

When the ultracentrifugation is continued for a longer period of time, the macromolecule [M] and the macromolecular-bound preservative $[M_nP]$ sediments (Garrett & Miller, 1965). The assayed concentration of the aqueous solution between the cream of oil particles and the sedimented macromolecules should represent the total concentration, μ'_{aq}, of unbound surfactant in the aqueous phase in all its ionic forms (Klotz, 1946, 1953; Goldstein, 1949).

From the knowledge of P_{aq} (equation 20), [M] (equation 21) and μ'_{aq}, the ratio r of preservative bound to macromolecule in the aqueous phase can be determined

$$r = [PM_n]/[M] = (P_{aq} - \mu'_{aq})/f\,[M]_T \quad .. \quad .. \quad (22)$$

The ratio r/μ' plotted against r permits the evaluation of n and K as given in equation 3.

Since the dissociation constant, K_a (equation 8) of the preservative is readily obtainable and the $[H^+]$ concentration can be obtained by pH measurement,

$$\mu_{aq} = \mu'_{aq}\{1/(1 + K_a/[H^+]\} \quad .. \quad .. \quad .. \quad (23)$$

from the statement of equation 9 where μ is the concentration of undissociated preservative in the aqueous phase. These procedures are schematically shown in Fig. 3.

Sufficient parameters are now available to estimate the apparent intrinsic partition coefficient k of the complex emulsion system

$$k = (P_T - \mu'')/\mu_{aq} = (P_T - \mu'_{aq})/\mu'_{aq}\{1/(1 + K_a/[H^+]\} \quad .. \quad .. \quad (24)$$

which is valid for the apparent oil/water volume ratio q. If the emulsion is diluted by an aqueous solution containing the same equilibrium concentration of surfactant, it is reasonable to assume that the apparent intrinsic partition constant k will be invariant for various q values.

When the stability of the preservative is examined to obtain the necessary degradation rate constant, k, and the minimum inhibitory concentration of the preservative, μ, is known, all the necessary parameters, viz. [M], n, k, K, q, k′, K_a and $[H^+]$ are available to predict the necessary initial preservative concentration $(P_T)_0$ for the complex system as in equations 19a and b.

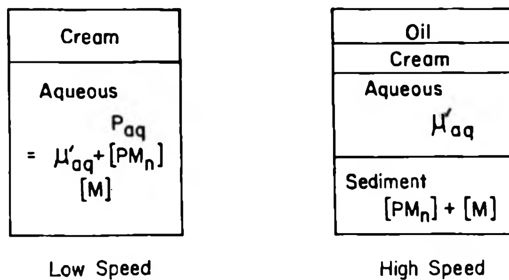

FIG. 3. Experimental determination of the apparent distribution coefficient, k, in a complex emulsion system. On low speed centrifugation, (a) assay the total concentration of macromolecule, [M], in the continuous phase as a function of the total macromolecule concentration, $[M_T]$, i.e. $[M] = f[M_T]$; (b) assay the total preservative bound and unbound to the equilibrium surfactant in the aqueous phase $P_{aq} = \mu'_{aq} + [PM_n]$, (c) On high speed centrifugation the macromolecule and macromolecular bound preservative $[M_nP]$ sediments and the total concentration, μ'_{aq}, of unbound surfactant in the aqueous phase can be assayed in all its ionic forms. (d) From the obtained P_{aq}, μ'_{aq} and [M], the ratio r can be calculated. $r = [PM_n]/[M] = (P_{aq} - \mu'_{aq})/f[M_T]$. (e) From the plot r/μ'_{aq} against r, obtain n and k since $r/\mu'_{aq} = n/K - (1/K)r$. (f) From the dissociation constant K_a and the $[H^+]$, $\mu_{aq} = \mu'_{aq}\,[1/(1 + K_a/[H^+])]$. (g) Thus $k = (P_T - \mu'_{aq})/\mu_{aq} = (P_T - \mu'_{aq})/\mu'_{aq}[1/1 + K_a/[H^+])]$, the apparent distribution coefficient of the complex system.

This method of evaluation should permit a more accurate estimation of the operative partition coefficient k, by the use of an actual emulsion system, than was obtained previously by simple partition between two discrete phases (Garrett & Woods, 1953).

Other testing procedures for evaluation of the physico-chemical factors affecting stability have been recently reviewed (Wedderburn, 1964).

The concept of the minimum inhibitory concentration as related to combinations of preservatives. The basic presumption of this development is that a definite concentration of preservative in its active form must be in solution in the aqueous phase, i.e. there must be a minimum inhibitory concentration, μ, of the biologically active species of the preservative to inhibit the growth of a specific micro-organism. For

reasons of toxicity and economy, the preservative concentration should not exceed the value necessary to maintain μ.

If it is presumed that structural modification of a basic function responsible for preservative action merely modifies μ for a particular substituted molecule and if it is presumed that preservative action of combinations of such preservatives is merely additive on the basis of equivalent potency, then there is no valid reason for using preservative combinations against a specific micro-organism. The choice is then purely on the basis of a potency/toxicity ratio with proper consideration of cost and the previously discussed preservative availability in the formulation to be considered as in equation 19.

This additivity of equivalent potencies is reasonable for substituted phenols and parabens and lends credence to this postulate (Littlejohn & Husa, 1955, Schimmel & Husa, 1956). The minor 20% variations can be easily explained on the basis that minor physico-chemical factors were not carefully controlled.

This is also verified from the fact that when combinations of antibacterials are evaluated on a kinetic basis against a single micro-organism, additivity of inhibitory rate constants can be demonstrated (Garrett & Brown, 1963). The use of the term synergism is generally merely a matter of improper definition of what constitutes additivity (Garrett, 1958). Unwarranted claims for the greater efficacy of antibiotic combinations have been criticised (Garrett, 1957).

Rationales for the use of combinations. The rationales for combinations are that the spectrum of activity can be increased; that the physiologically harmful effects of a dose of one preservative alone giving an equivalent effect may be averted; that the development or modification of the resistance of an organism to one preservative alone may be prevented; that response may exceed prediction from the separate preservative action or from any concentrations of one preservative alone; that convenience of administration of smaller preservative amounts or economic savings may result.

The possible presence of various micro-organisms causing spoilage, each with a different μ value for a given preservative, may warrant the use of preservative combinations. If incompatibilities such as complexing and precipitations do not exist, the necessary amounts of each preservative may be predicted on the basis of the use of equation 19.

The choice of a wide-spectrum of preservatives to inhibit different varieties of organisms must be based on criteria of specificity, cost and toxicity.

When the limiting factor for effective action is the solubility of a single preservative in the aqueous phase, the use of combinations to achieve the necessary overall minimum inhibitory concentration is warranted.

A simple and logical terminology (Garrett, 1958) to classify preservative action against a single organism and consistent with literature usage may be based on two *a priori* postulates of combined action: (*a*) additivity, where the combined response is additive with respect to the separate

responses of the components and (*b*) equivalence, in which the components act in the same manner with the same dose response curve, separately or in combination, except for a difference in the weight of an arbitrarily defined therapeutic "unit dose".

Additivity is expected on the basis that two preservatives act independently and do not affect each other's mode, degree, or efficacy of action.

Equivalence is expected on the basis that different amounts of the same or equivalent drugs or potency factors are combined. The use of this criterion would exclude from classifications of synergism or antagonism those anomalous responses of combinations where the components differ only in the dilution of the same or similarly acting potency factor. Since dose response correlations are too frequently nonlinear, nonadditivity of responses could classify combinations of dose of the same preservative as antagonistic or synergistic.

Only under special conditions could "additivity" or "equivalence" criteria give the same response for a combination, the least probable circumstance of a linear dose-response curve (Garrett, 1958).

A logical classification of combined drug response (Garrett, 1958) based on fulfilment or nonfulfilment of these postulates, would be "less than additive" (group 1), "additive" (group 2) and "more than additive" (group 3). The three categories in each group would be "less than equivalent" (A types), "equivalent" (I types) and "more than equivalent" (S types).

This classification has certain conveniences. For example, if the response-equivalent concentrations are equal in cost, all S types are more economically used as the combination, all A types as the single preservative. All I types have a high probability that the constituent drugs will have the same mechanism of action, whereas S and A types must differ; in the former, alternate metabolic pathways may be blocked, and in the latter, the preservatives may compete, form inactive compounds or complexes or activate alternate metabolic pathways.

Consideration of proper choice of response to characterize combined preservative action. The test for "equivalence" needs a knowledge of the response as a continuous function of dose. Jawetz & Gunnison (1952) have criticized the minimum inhibitory dose methods on the basis that they presuppose a linear relation between inhibitory action of each preservative and its dose.

Additivity of kill is not the same as additivity of rates of kill. Mathematically (Garrett, 1958) rates of logarithmic death due to combined preservative action could equal the sum of the rates due to the separate preservative, whereas the fractional kill at a particular time would not equal the sum of the fractional kills of the separate drugs, and vice versa.

Logarithmic viable count-time curve slopes that are functions of rate constants are good criteria of preservative action. A better criterion would be the rate constants themselves, determined for rates of kill and inhibition of growth of micro-organisms. These could serve as proper responses for classification and evaluation of combined antibiotic action.

The magnitude of these responses should be a continuous function of a dose.

This philosophy of approach has been applied recently (Garrett & Brown, 1963; Brown & Garrett, 1964; Garrett & Miller, 1965) with specific reference to chloramphenicol and tetracycline.

The generation rate constants k from

$$N = N_0 e^{kt} \qquad (25)$$

$k > O$ for viable counts, N, of *E. coli* growth have been shown to be linearly dependent on the concentration, A, of the antibiotics tetracycline or chloramphenicol, or both (Garrett & Brown, 1963, Brown & Garrett, 1964)

$$k = k_0 - k_A A \qquad (26)$$

It has also been shown that the total counts by the Coulter Counter and the viable counts by the colony technique are equivalent in the presence of these antibiotics (Garrett & Miller, 1965) and the mode of action is inhibitory for $k > O$.

It follows that when $k = O$, the concentration $A_{k=O}$ may serve as a preliminary estimate of the minimum inhibitory concentration, μ_{EST}, and from equation 26

$$\mu_{EST} = A_{k=O} = k_0/k_a \qquad (27)$$

The equation 19c accounts for the consumption and degradation of the antibiotic so that the value of $(P_T)_0$ to be chosen will depend on the time, t, for which bacteriostasis by the $\mu \sim \mu_{EST}$ of equation 27 is required.

Of course, a value of μ greater than μ_{EST} may be needed to ensure permanent inhibition since bacteriostasis may not be adequate for satisfactory preservative action. A value of the minimum preservative concentration μ, necessary for insertion into equation 19c may have to be chosen on the basis of need of bactericidal activity so that the growth rates never recover to positive values.

The dependence of *E. coli* generation rate constants on antibiotic concentration (equation 26) has been shown (Garrett & Miller, 1965) to be independent of the inoculum size over a range of 10^3 micro-organisms/ml (i.e. 10^3–10^6). If all preservatives act similarly, the necessary minimum inhibitory concentration will be independent of the number of contaminating micro-organisms. It follows that since the purpose of a preservative is to inhibit the growth of even one micro-organism, the $\mu_{EST} = A_{k=O}$ obtained from a kinetic analysis (equations 25–27) of many micro-organisms may be a satisfactory preliminary estimate for such inhibition.

If the necessary minimum preservative concentration is not independent of inoculum size, it will be necessary to determine this dependence and provide that amount, μ, which will satisfactorily inhibit or kill the largest number of micro-organisms anticipated in the preparation. Fortunately, only small numbers of contaminant micro-organisms are anticipated in practice.

In a given system at a given temperature, it has been shown that equations 25–27 hold for variously substituted chloramphenicols (Garrett Miller & Brown, 1966) and for tetracycline. In the instance of tetracycline and chloramphenicol, the inhibitory rate constants are k_A for tetracycline, k_T, and for chloramphenicol, k_C.

When additivity of action of the combined antibiotic concentrations on rates of *E. coli* growth was postulated, it was predicted for our system that the overall rate constant for growth would be

$$k = k_0 - k_T T - k_C C \qquad (28)$$

in accordance with the extension of equation 26 where T and C are the concentrations of chloramphenicol and tetracycline respectively.

The exponential change of *E. coli* viables (Garrett & Brown, 1963), and thus *E. coli* totals (Garrett & Miller, 1965), was invariant with varying ratios of chloramphenicol to tetracycline, so calculated that the potency was equivalent on the basis of equation 28, i.e. 7·5 weight units of chloramphenicol considered equipotent to 1 weight unit of tetracycline hydrochloride in our system. This was confirmation of the additivity of the antimicrobial effects for these two antibiotics. The equipotent additivity also held as expected when the overall rate constant k = O so that the additivity of weighted contributions to the minimum inhibitory concentration was also verified. A reduction in the rate of growth of the organism in the presence of equipotent antibiotic mixtures (e.g. "kill" in the presence of weighted amounts in the combination predicted to just give complete inhibition) would have indicated synergism. Conversely, an increase in the rate with the mixtures compared to that for either antibiotic alone on the basis of equipotency would have indicated antagonism (i.e. some net growth in the presence of weighted amounts in the combination predicted to just give complete inhibition).

Of course, minimum inhibitory concentrations based on bacteriostatic action may not be adequate for satisfactory preservative activity since bactericidal activity is also desired. The death of all viable organisms in a preparation is a proper function of a proper perservative. The work with tetracyclines and chloramphenicols just described serve only to demonstrate procedures applicable to estimate such minimum inhibitory concentrations for various preservatives against a single organism on the basis of a kinetic model. Other kinetic models which may be applicable have been discussed in detail (Garrett, 1958). The prediction of optimum combinations of preservatives may be made on the basis of these evaluations of combined biological effects and the physical chemical factors which have been detailed.

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References

Allawala, N. A. & Riegelman, S. (1953). *J. Am. pharm. Assoc., Sci. Ed.*, **42**, 267–275, 396–401.
Anton, A. H. (1960). *J. Pharmac. exp. Ther.*, **129**, 282–290.
Anton, A. H. (1961). *Ibid.*, **129**, 291–303.
Bean, H. S., Heman-Ackah, S. M. & Thomas, J. (1965). Symposia Preprints, 112th Annual Meeting of the American Pharmaceutical Association, Detroit, Michigan.
Bennett, E. O. (1959). *Adv. appl. Microbiol.*, **1**, 123–140.
Bjerrum, U. (1941). *Metal Amine Formation in Aqueous Solution*, Copenhagen: Hasse.
Brown, M. R. W. & Garrett, E. R. (1964). *J. pharm. Sci.*, **53**, 179–183.
Calvin, M. & Melchior, N. C. (1948). *J. Am. chem. Soc.*, **70**, 3270–3273.
Cook, A. M. (1960). *J. Pharm. Pharmac.*, **12**, 9T–18T.
Garrett, E. R. (1957). *Post-grad. Med.*, **22**, 427–428.
Garrett, E. R. (1958). *Antibiotica Chemother.*, **8**, 8–20.
Garrett, E. R. (1962). *J. pharm. Sci.*, **51**, 35–42.
Garrett, E. R. & Brown, M. R. W. (1963). *J. Pharm. Pharmac.*, **15**, *suppl.*, 185*T*–191*T*.
Garrett, E. R. & Miller, G. H. (1965). *J. pharm. Sci.*, **54**, 427–431.
Garrett, E. R., Miller, G. H. & Brown, M. R. W. (1966). *Ibid.*, **55**, 593–600.
Garrett, E. R. & Woods, O. R. (1953). *J. Am. pharm. Ass., Sci. Ed.*, **42**, 736–739.
Goldstein, A. (1949). *Pharmac. Rev.*, **1**, 102–165.
Guttman, D. & Higuchi, T. (1957). *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 4–10.
Higuchi, T. & Lach, J. L. (1954a). *Ibid.*, **43**, 465–470.
Higuchi, T. & Lach, J. L. (1954b). *Ibid.*, **43**, 524–527.
Higuchi, T. & Zuck, D. A. (1953). *Ibid.*, **42**, 132–138.
Jacobs, S. E. (1960). *J. Pharm. Pharmac.*, **12**, 9T–18T.
Jawetz, E. & Gunnison, J. B. (1952). *J. Am. med. Ass.*, **150**, 693–695.
Job, P. (1928). *Annls Chim.*, **9**, 113–203.
Klotz, I. M. (1946). *Archs Biochem.*, **9**, 109–117.
Klotz, I. M. (1953). In *The Proteins*, **1B**, Neurath, H. & Bailey, K. New York: Academic Press.
Krüger-Thiemer, E., Diller, W., Dettli, L., Bünger, P. & Seydel, J. (1964). *Antibiot. Chemotherapia Fortschr.*, **12**, 171–193.
Littlejohn, O. M. & Husa, W. J. (1955). *J. Am. pharm. Ass., Sci. Ed.*, **44**, 305–308.
MacCallister, R. V. & Lisk, R. J. (1951). *Analyt. Chem.*, **32**, 609–610.
Martell, A. E. & Frost, A. E. (1950). *J. Am. chem. Soc.*, **72**, 3743–3746.
Miyawaki, G. M., Patel, N. K. & Kostenbauder, H. B. (1959). *J. Am. pharm. Ass., Sci. Ed.*, **48**, 315–318.
Navarre, de, M. G. (1962). *Chemistry and Manufacture of Cosmetics*, **1**, 2nd Edn. Princeton: Van Nostrand.
Patel, N. K. & Kostenbauder, H. B. (1958). *J. Am. pharm. Ass., Sci. Ed.*, **47**, 289–293.
Pisano, F. D. & Kostenbauder, H. B. (1959). *Ibid.*, **48**, 310–314.
Rahn, O. & Conn, J. E. (1944). *Ind. Engng Chem.*, **36**, 185–187.
Reddish, G. F. (1957). *Antiseptics, Disinfectants, Fungicides and Sterilization*, 2nd Edn. Philadelphia: Lea & Febiger.
Schimmel, J. & Husa, W. J. (1956). *J. Am. pharm. Ass., Sci. Ed.*, **45**, 204–208.
Sykes, G. (1958). *Disinfectants and Sterilization.* Princeton: Van Nostrand.
Tice, L. F. & Barr, M. (1959). *Am. Perfumer Arom.*, **73**, 20–23.
Vosburgh, W. C. & Cooper, G. R. (1941). *J. Am. chem. Soc.*, **63**, 437–442.
Wedderburn, D. L. (1964). In *Advances in Pharmaceutical Sciences*, Bean, H. S., Beckett, A. H. & Carless, J. E., New York: Academic Press.
Wyss, O. (1948). *Adv. Food Res.*, **1**, 373–393.

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The effect of pH and temperature on the lysis of yeast cells by cationic dyes and surfactants

J. C. RIEMERSMA

Conductivity measurements in yeast suspensions, to which a stepwise increasing quantity of a cytolytic reagent was added, provided evidence towards explaining possible mechanisms of cytolysis by cationic dyes and by cationic surfactants. These lytic cations are bound to the cell surface. If uptake exceeds a critical amount, the permeability barrier of part of the yeast cells in the suspension is destroyed. The "threshold" amount of lytic agent was proportional to the quantity of yeast in the suspension, and was pH- and temperature dependent in a way which suggested an interaction with anionic membrane lipids. There were similarities between the cationic dyes, Toluidine Blue and Azure A, and the cationic surfactants, cetrimide and cetylpyridinium chloride, in their cytolytic effect on yeast cells. A mechanism of membrane breakdown is discussed.

SEVERAL authors have attributed the bactericidal action of long-chain alkylammonium compounds to their protein-denaturing properties which might result in an inactivation of cellular enzymes (Valko, 1946). Others have directed attention to the cytolytic properties of cationic surfactants. Hotchkiss (1946), Salton (1951) and others have attributed antiseptic action to the membrane-disorganizing effects of these compounds. The observed influence of surfactant alkyl chain length on bactericidal and cytolytic effectiveness suggests an interaction with membrane lipids (Cella, Eggenberger, Noel, Harriman & Harwood, 1952; Ross, Kwartler & Bailey, 1953; Ross & Silverstein, 1954; Hooghwinkel, De Rooij & Dankmeijer, 1965). Surfactants can no doubt interact with enzymes and other cell proteins, but the most likely mechanism of cytolysis appears to involve an interaction with the permeability barrier of the cell (Schulman, Pethica, Few & Salton, 1955; Pethica, 1958; Newton, 1958).

The present paper discusses lysis in yeast cells (*Saccharomyces cerevisiae*) by two cationic dyes and two cationic surfactants. Extensive use was made of conductivity measurements, because a separation of cells and extracellular fluid is not required; furthermore, leakage of ions and cytolysis are registered as conductivity changes as they occur. A rapid assessment of the influence of such variables as temperature and external pH on cytolysis can be made.

Experimental

Materials. Commercial baker's yeast (fresh Koningsgist, Delft), was washed by suspension in distilled water, centrifugation and resuspension. To obtain cells in a reproducible "starved" condition, air was bubbled through the suspension overnight to exhaust endogenous substrates. After aeration the yeast was freed from debris by fourfold centrifugation and resuspension. Centrifugation periods were for only 1–2 min at 3000 rpm to keep many of the cells suspended; these were removed with the debris. The remaining cell suspension was centrifuged at 3000 rpm for 20 min. The packed cells were used to prepare a stock suspension in distilled water, containing 35·5 g yeast/100 ml ("35·5% suspension").

From the Laboratory of Medical Chemistry, University of Leiden, Holland.

Cetylpyridinium chloride and cetyltrimethylammonium bromide (cetrimide), of high purity (>95%), were supplied by F. W. Berk, London. Sodium dodecylsulphate was supplied by Unilever Research Laboratory, Vlaardingen, through the courtesy of Dr. K. van Senden. Toluidine Blue O and Azure A as chlorides, supplied by National Aniline Division, Allied Chemical Co. were used because they were relatively pure as shown by paper chromatography (Persijn, 1961) and conductimetric analysis.

Apparatus. A Philips PR 9501 conductivity meter was used. Reagents were added from a Metrohm piston burette to a magnetically stirred yeast suspension (total volume 32 ml) in a double-walled glass vessel connected with a thermostat. After each reagent addition a time-interval (1 or 2 min respectively) was allowed to elapse before the conductivity reading. By intensive stirring a local excess of reagent was avoided.

Analysis of the extracellular fluid. At intervals during the determination of a conductivity curve a small known volume of suspension was rapidly filtered through a Millipore filter (HA, 0·45 μ pore size). Potassium in the extracellular fluid was determined by flame photometry. An estimate of extracellular nucleotide concentration was obtained by measuring the absorbance at 260 mμ. Chloride ions were titrated

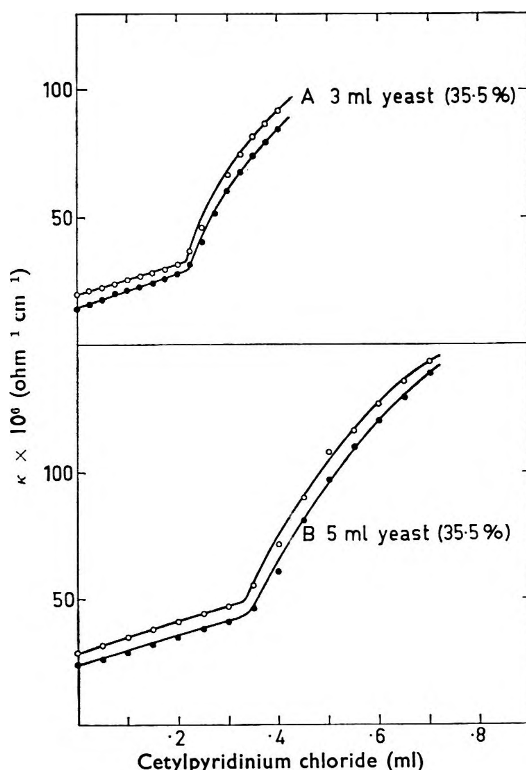

FIG. 1. Specific conductance changes in a yeast suspension (21°) during stepwise addition of 0·01 M cetylpyridinium chloride. A. 3 ml 35·5% yeast + 29 ml water. B. 5 ml 35·5% yeast + 27 ml water.

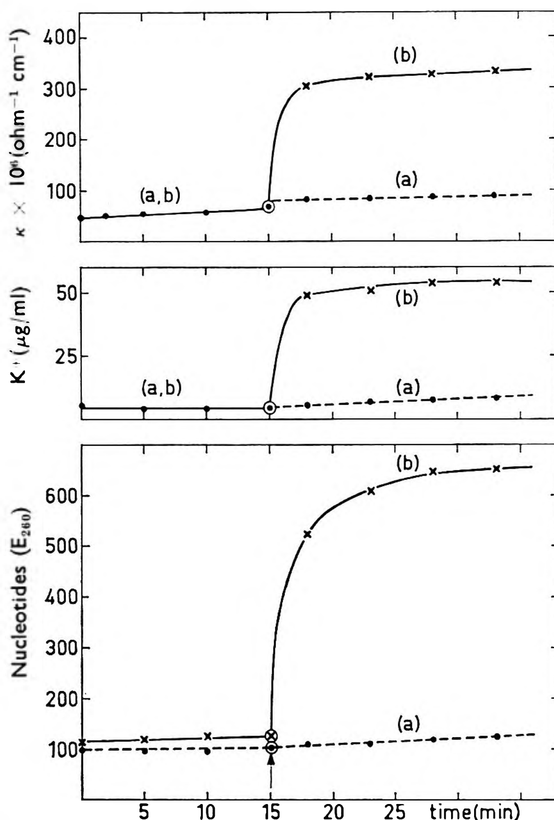

FIG. 2. Specific conductance, potassium concentration, and nucleotide concentration in the extracellular medium as a function of time. The suspension contained 50 ml 35·5% yeast + 110 ml water at 20°. At t = 15 min (*a*) 2 ml 0·01M cetrimide; (*b*) 5 ml 0·01M cetrimide were added (previously determined critical quantity: 3·4 ml 0·01M cetrimide).

conductimetrically, in an 80% ethanol medium, with 0·001M silver nitrate in 80% ethanol. Cationic surfactants were determined with the Bromophenol Blue method, as described by Auerbach (1943) and modified by Van Steveninck & Maas (1965). A similar method was used to determine dodecylsulphate, with the basic dye Azure A to form a coloured complex. This complex was extracted from an aqueous solution containing 0·01M sulphuric acid by means of 1,2-dichloroethane.

Results

Conductivity measurements were made in yeast suspensions to which successive quantities of 0·01M cetrimide were added, as specified in the legend of Fig. 1. With increasing surfactant additions, a phase of relatively minor conductivity changes was followed by a more rapid increase. By taking samples of suspension and analysing the extracellular fluid after Millipore filtration, the break of the conductivity curve

was found to occur at a "critical" surfactant addition beyond which there was rapid release of cellular potassium ions and of nucleotides.

Additions below the critical quantity of surfactant caused a negligible immediate release of intracellular constituents and a slightly increased continuous leakage of potassium ions and of nucleotides.

Fig. 2 shows conductivity changes as a function of time, after one addition of cetrimide in an amount below the cytolytic threshold (*a*) and above this threshold (*b*). The yeast suspension (*a*) showed negligible ion losses from the cells, mostly due to an increased rate of steady leakage. In (*b*) there was a sizeable and relatively rapid ion loss from the cells which approached a plateau. Cetylpyridinium chloride behaved similarly.

From samples of extracellular medium after Millipore filtration it could be shown that there was no equilibrium surfactant concentration

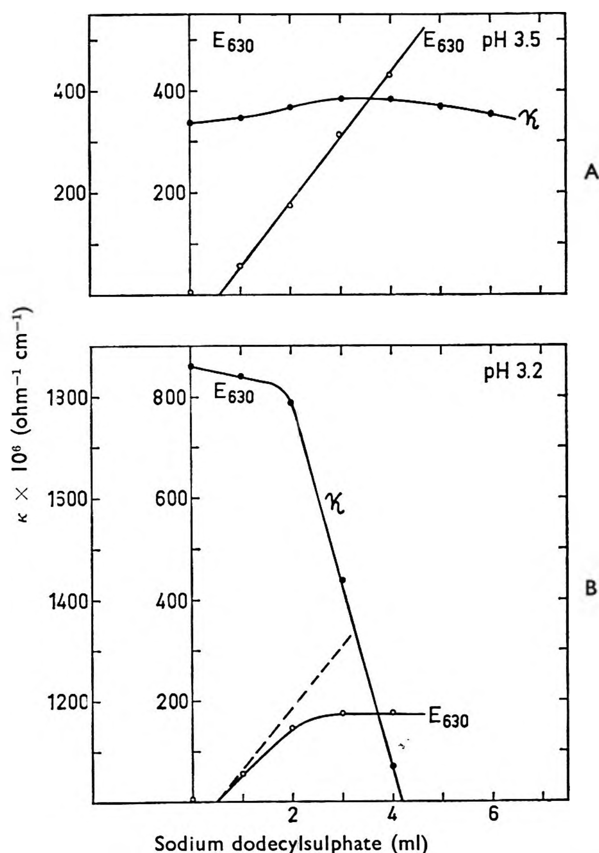

FIG. 3. Specific conductance (κ) changes in a yeast suspension (21°) during step-wise addition of 0·05M sodium dodecylsulphate. The lines for E_{630} indicate the rise in dodecylsulphate concentration in the extracellular medium (determined as a complex with Azure A having an absorption maximum at 630 mμ), as a function of total sodium dodecylsulphate added. A. pH 3·5, 28 ml 35·5% yeast + 100 ml water. B. pH 3·2, 28 ml 35·5% yeast + 100 ml water. Case B, only, shows surfactant uptake and cytolysis.

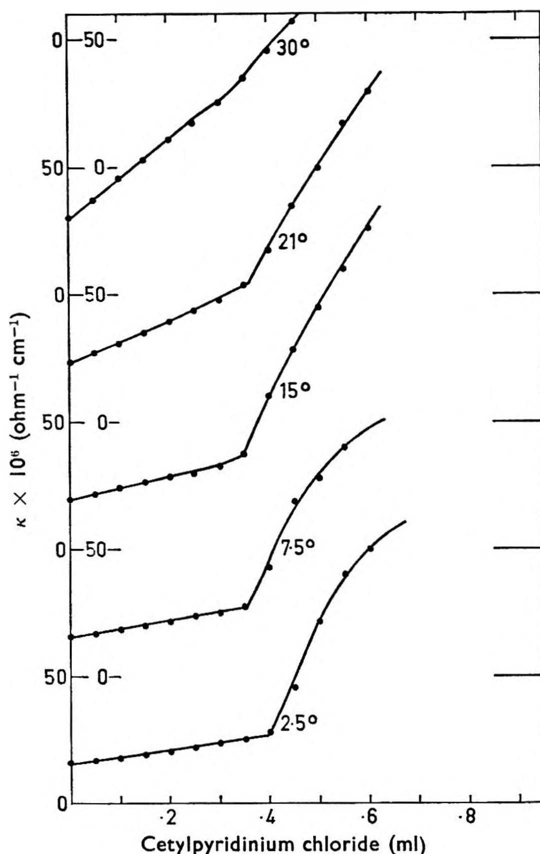

FIG. 4. Temperature influence on the course of conductance change during stepwise addition of 0·01M cetylpyridinium chloride to a yeast suspension (5 ml 35% yeast + 27 ml water; temperatures in °C).

in the medium; all the added surfactant ions were bound by the cells. Determinations of chloride and bromide ions in the medium showed that these ions were not taken up by the cells during surfactant addition.

In experiments with varying quantities of yeast in the same volume of suspension, the critical cytolytic surfactant quantity increased linearly with the quantity of yeast (Fig. 1). The slope of this line gives an estimate of the quantity of surfactant taken up before cytolysis began. This value is 1·8 μmole cetylpyridinium chloride per g fresh yeast, 1·9 μmole/g for tetradecylpyridiniumchloride, and 1·9 μmole/g for cetrimide.

The same sequence of conductivity changes was observed with Toluidine Blue and Azure A as with the cationic surfactants, but the break in the conductivity curve corresponded to a higher uptake of reagent. Again the whole of the added lytic substance was bound by the cells. Conductivity changes ran parallel with increased release of intracellular potassium to the medium.

The threshold cytolytic quantity of dye showed a linear dependence on the yeast quantity; 5·7 μmole Azure A/g yeast and 5·6 μmole Toluidine

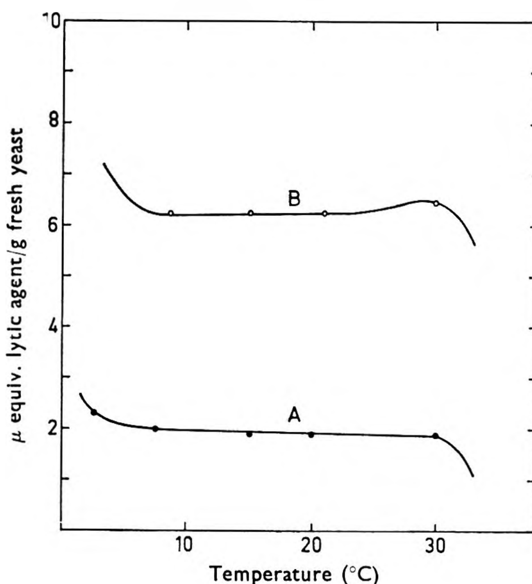

FIG. 5. Cytolytic threshold quantity as a function of temperature, for cetylpyridinium chloride and Azure A, on the basis of specific conductance curves. A. 0·01M cetylpyridinium chloride, added to a suspension containing 5 ml 35·5% yeast + 27 ml water. B. 0·05M Azure A added to a suspension of 7 ml 35·5% yeast + 25 ml water.

Blue/g yeast respectively were required to induce rapid cytolysis. Analysis of the extracellular medium showed that no free dye cations remained in solution, hence the total dye quantity added was bound by the cells.

The similar effects of dye and surfactant cations suggest that, in cytolysis, interactions with charged groups at the cell surface may be involved. Varying the cell surface charge by the external pH should according to this hypothesis have an effect on cytolysis. Conductivity experiments were undertaken with the initial pH of the cell suspension at 2·8, 3·0, 3·2, 3·5, 4·0, 5·0, 6·0, 6·5, 7·0, by the addition of 0·1N hydrochloric acid or tris (hydroxymethyl)aminomethane. Over the pH range 3·5 to 6·0 the cytolytic threshold quantity of cetylpyridinium chloride or cetrimide did not change with extracellular pH. At a pH of 3·2 or lower and at pH 6·5 and higher, small quantities of the surfactants cause rapid cytolysis.

For Toluidine Blue and Azure A, at pH 3·5 and pH 7·0 the critical quantity was shifted to zero, so that very small dye quantities were sufficient to cause cytolysis.

An anionic surfactant, sodium dodecylsulphate was similarly examined. At pH values above 3·5 no cytolysis occurred, nor were dodecylsulphate ions taken up by the cells. At a pH of 3·2, the conductivity curve showed a break corresponding to the point where rapid cytolysis occurred while there was a large uptake of dodecylsulphate ions (Fig. 3). Uptake was far from quantitative; in the medium a large equilibrium concentration of dodecylsulphate was found—in contrast to the cationic surfactants.

At 30° and higher the yeast cells become extremely sensitive to surfactants. Between 10° and 30° the threshold quantity is constant, though

continuous ion leakage from the cells occurs more rapidly with higher temperatures (Fig. 4). Below 10° an increased quantity of surfactant was needed to cause cytolysis. Similar phenomena were found with the dyes; here at 2–3° cytolysis was completely inhibited (Fig. 5).

Discussion

The two cationic surfactants and two dyes, although differing in molecular structure, exhibit striking similarities in their cytolytic effects. The cation is strongly and completely bound by the yeast cells and, at a critical quantity bound per gram cells, an extensive release of cytoplasmic constituents occurs. But with an anionic surfactant lytic effects were seen only at low pH. Neutral substances such as lower alcohols (C_1, C_2, C_3, C_4) also cause lysis in yeast cells but ion release is relatively slow and gradual (Blennemann, Janocha, Keller & Netter, 1963).

It appears that quaternary ammonium surfactants have only a slight disruptive effect on the yeast cell membrane below the critical quantity corresponding to the break in the conductivity curves. The result is a slight acceleration of the steady ion leakage which is always found in yeast cell suspensions, and which may be ascribed to membrane penetration by surfactant molecules (cf. Booij & Bungenberg de Jong, 1956). As Fig. 2 shows, beyond the break in the conductivity curve a different type of ion release occurs. At this point a large quantity of intracellular ions are released in a relatively short time. The time-course of the changes in extracellular potassium and nucleotides suggests that some cells suffer complete membrane breakdown and thus release their total ion content to the medium. For benzalkonium chloride, Sharff & Maupin (1960) found a close agreement between the curves representing potassium release and the percentage of cells stainable by Nile Blue. Potassium loss may thus be due to a complete membrane breakdown of some of the cells in the suspension (all-or-none effect), although this would have to be confirmed by further counting experiments.

To obtain complete lysis of all the cells present in a yeast suspension, 40–100 times as much surfactant as is necessary to reach the cytolytic threshold is required. This may be due to the variation of individual cells in their resistance to surfactant action. Another factor is probably even more important, namely that after passing the critical surfactant quantity the content of lysed cells competes with the membrane of the remaining intact cells for the added surfactant.

The cytoplasm of the yeast cell is probably surrounded by a bimolecular lipid membrane (Gorter & Grendel, 1926; Danielli & Harvey, 1935; see also Booij & Bungenberg de Jong, 1956; Davson, 1962) which functions as a permeability barrier. The membrane is in turn surrounded by a relatively thick cell wall which confers osmotic stability; hence the cells can be put in distilled water without lysis. The main constituents of the membrane are known to be the polysaccharides glucan and mannan. Some protein and lipid constituents are also present (Northcote & Horne, 1952; Nickerson, 1964).

The anionic groups which interact with cytolytic cations are unlikely

to be located in the extraneous cell wall. It would be hard to explain the occurrence of membrane breakdown and the instantaneous release of cellular constituents following the accumulation of lytic cations in the cell wall. A more plausible hypothesis is that the anionic groups involved are themselves part of the lipid permeability barrier of the cell. Observations on the protection afforded by certain metal ions against cytolysis by cationic surfactants also agree with this.

Although proteins may also be part of the membrane-complex in a wider sense, the relative pH-independence and temperature-independence of the critical quantities of dye and surfactant, necessary for cytolysis, argue against a major role of protein in the binding of lytic cations.

The critical quantities of the four cationic reagents may be compared with the estimated number of lipid molecules in the cell membrane (Van Steveninck & Booij, 1964). Assuming that the membrane is a lipid bilayer, the number of lipid molecules in the membrane is approximately *twice* the number of polar groups of a representative phospholipid that can be accommodated at the outward surface of the membrane. Per gram yeast approximately 10^{10} cells are present, while the cell surface is about $120\,\mu^2$ ($= 120 \times 10^{-12}$ m^2). Assuming a cross-section of lecithin, a representative phospholipid, of 40 Å^2 ($=40 \times 10^{-20}$ m^2), we arrive at 6×10^8 molecules of lecithin per cell. The number of membrane phospholipids per gram yeast is 6×10^{18} or approximately 10 μmoles/g. The cross-section of 40 Å^2 represents a lower limit, found only in monolayers of saturated phospholipids. Depending on the acyl groups of the various phospholipids in the membrane a more or less "condensed" state of the lipids can be expected (Van Deenen, 1965). At a cross-section of 60 Å^2, 6·7 μmoles/g yeast would be present as membrane lipids, which is about 4 times the quantity of cationic surfactant required for cytolysis.

The composition of the yeast cell membrane is not known, but figures have been obtained for the phospholipid composition of the whole yeast cell. An analysis of the kind of yeast used in our experiments, carried out by F. A. Deierkauf in this laboratory, gave the following quantities of lipid (μmoles P) per gram yeast: phosphatidylcholine + phosphatidyl ethanolamine 51·8; phosphatidyl serine 5·2; mono-phosphoinositide 12·6; di-phosphoinositide 0·7; phosphatidic acid 1·8; unidentified 1·8; giving a total of 73·9 μmoles P.

Given the relative importance of anionic lipids in the lipid composition of the yeast cell, a role of lipid anions in the binding of lytic cations may be considered likely. Anionic phosphatides are not only a major lipid component in yeast but figure predominantly in the chemical composition of the protoplast membrane of other micro-organisms such as *Micrococcus lysodeikticus* (Gilby, Few & McQuillen, 1958; Wolfe, 1964). A negatively charged membrane surface could account for the quantitative and immediate uptake of dye or surfactant cations as observed. Membrane breakdown could be envisaged as a special case of lipid-surfactant interactions as have been studied already with isolated lipids *in vitro*.

Micelles of phosphoinositides in water, obtained by sonic dispersion, consist of concentric layers of lipid lamellae. They have been found to

be transformed into globular lipid micelles by the action of cetrimide (Thompson & Dawson, 1964; *cf.* Bangham, 1963). The same type of phase transition may be responsible for the disruption of a cell membrane, composed in part of anionic lipids, under the influence of cetrimide and related lytic substances. Such a mechanism would imply a total membrane breakdown once a critical quantity of surfactant cations has been taken up. The observations regarding a critical surfactant quantity required for beginning all-or-none cytolysis strongly suggest such a mechanism. The relative pH-independence of the critical quantity of lytic agent required for cytolysis, as well as the observed dependence of cytolytic behaviour on the temperature, are both in agreement with the idea that anionic lipids are responsible for the net charge of the membrane, and are involved in an interaction with the membrane-disrupting agents discussed.

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References

Auerbach, M. E. (1943). *Analyt. Chem.*, **15**, 492–493.
Bangham, A. D. (1963). *Adv. Lipid Res.*, **1**, 65–104.
Blennemann, H., Janocha, S., Keller, H. & Netter, H. (1963). *Hoppe-Seyler's Z. Physiol. Chem.*, **331**, 164–179.
Booij, H. L. & Bungenberg de Jong, H. G. (1956). *Protoplasmatologia*, I, **2**, p. 144. Vienna: Springer.
Cella, J. A., Eggenberger, D. N., Noel, D. R., Harriman, L. A. & Harwood, H. J. (1952). *J. Am. chem. Soc.*, **74**, 2061–2062.
Danielli, J. F. & Harvey, E. N. (1935). *J. cell. comp. Physiol.*, **5**, 483–494.
Davson, H. (1962). In *Symposium on the plasma membrane, Circulation*, **26** (Supplement), 1022–1037.
Gilby, A. R., Few, A. V. & McQuillen, K. (1958). *Biochem. biophys. Acta*, **29**, 21–29.
Hooghwinkel, G. J. M., De Rooij, R. E. & Dankmeijer, H. R. (1965). *Acta physiol. pharmac. neerl.*, **13**, 304–316.
Gorter, E. & Grendel, F. (1926). *Proc. K. ned. Akad. Wet. Sec. B.*, **29**, 318–20.
Hotchkiss, R. D. (1946). *Ann. N.Y. Acad. Sci.*, **46**, 479–493.
Newton, B. A. (1958). In *The Strategy of Chemotherapy*. Editors: Cowan, S. T. & Rowatt, E., pp. 62–93. Cambridge: University Press.
Nickerson, W. J. (1964). In *Cellular Membranes in Development*. Editor: Locke, M., pp. 291–297, New York: Academic Press.
Northcote, D. H. & Horne, R. W. (1952). *Biochem J.*, **51**, 232–236.
Persijn, J. P. (1961). *Stain Technol.*, **36**, 27–29.
Pethica, B. A. (1958). *J. gen. Microbiol.*, **18**, 473–480.
Ross, S., Kwartler, C. E. & Bailey, J. H. (1953). *J. Colloid Sci.*, **8**, 385–401.
Ross, S. & Silverstein, A. M. (1954). *Ibid.*, **9**, 157–165.
Salton, M. R. J. (1951). *J. gen. Microbiol.*, **5**, 391–404.
Scharff, F. G. & Maupin, W. C. (1960). *Biochem. Pharmac.*, **5**, 79–86.
Schulman, J. H., Pethica, B. A., Few, A. V. & Salton, M. B. J. (1955). *Progress in Biophysics*, **5**, 41–71.
Thompson, W. & Dawson, B. M. C. (1964). *Biochem. J.*, **91**, 237–243.
Valko, E. J. (1946). *Ann. N.Y. Acad. Sci.*, **46**, 451–478.
Van Deenen, L. L. M. (1965). *Progress in the chemistry of fats and other lipids*, **8**, part 1, p. 59, Oxford: Pergamon Press.
Van Steveninck, J. & Booij, H. L. (1964). *J. gen. Physiol.*, **48**, 43–60.
Van Steveninck, J. & Maas, M. (1965). *Rec. Trav. chim. Pays.-Bas Belg.*, **84**, 1166–1168.
Wolfe, L. S. (1964). *Can. J. Biochem.*, **42**, 971–988.

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Lowering of plasma triglycerides as a result of decreased free fatty acid mobilization

A. BIZZI, E. VENERONI, S. GARATTINI

3,5-Dimethylpyrazole and its metabolite 3-carboxy-5-methylpyrazole lower plasma FFA and decrease the level of plasma triglycerides. The metabolite given intravenously is more rapidly effective than 3,5-dimethylpyrazole. Plasma cholesterol and phospholipids as well as blood glucose were not affected by these acute treatments.

A RELATION between changes of lipid mobilization and alterations of plasma lipids in several animal species has been suggested by several authors. For instance, adrenaline, a potent releaser of free fatty acids (FFA), increases plasma triglycerides in dogs and man (Dury & Treadwell, 1955; Shafrir, Sussman & Steinberg, 1959; Carlson, Boberg & Högstedt, 1965). Adrenocorticotrophic hormone increases plasma triglycerides in rabbits (Woods, Freedman & Kellner, 1962). On the other hand, many drugs known to depress lipid mobilization have been described as hypolipaemic agents. Nicotinic acid decreases plasma triglycerides in rats (Jacobs, Grebner & Cook, 1965), and like salicylates (Alexander, MacDougall, Oliver & Boyd, 1959; Reid, 1961), ganglionic and adrenergic blocking agents (Hollister, Kanter, Powell & Henrich, 1957; Deming, Hodes, Baltazar, Edreira & Torosday, 1958) it reduces plasma cholesterol in man (Altschul, Hoffer & Stepnen, 1955). Recently, 3,5-dimethylpyrazole (DMP) was reported to decrease plasma FFA in a variety of experimental conditions (Gerritsen & Dulin, 1965a; Bizzi, Jori, Veneroni & Garattini, 1964).

This drug has since been shown to decrease lipolysis in adipose tissue (Gerritsen & Dulin, 1965b; Garattini & Bizzi, 1966; Bizzi & Garattini, 1966a) and the formation of triglycerides in the liver (Bizzi & Garattini, 1966b; Bizzi, Tacconi, Veneroni & Garattini, 1966).

These effects of DMP are probably mediated by an acid metabolite, 3-carboxyl-5-methylpyrazole (CMP) (Gerritsen & Dulin, 1965b) which has been isolated from the urine of animals treated with DMP (Smith, Forist & Dulin, 1965).

We report the effect of DMP and of its metabolite on the level of the plasma lipids (triglycerides, cholesterol and phospholipids) in rats.

Experimental

MATERIALS AND METHODS

Male Sprague-Dawley rats, from ALAL breeding, fasted overnight were used. DMP and its metabolite were given orally or intravenously, at doses and times indicated in the Tables. Plasma FFA levels were determined by Dole's method (1956) with minor modifications. A washing with 0·05% sulphuric acid according to Trout, Estes & Friedberg (1960)

From the Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, Milan, Italy.

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was adopted to avoid the interference of CMP in the titration. Lipids were extracted with chloroform-methanol 2:1, and washed with saline. The phosphorus of phospholipids was determined in th chloroform extracts according to Lowry, Roberts, Leiner & Farr (1954). The residual chloroform extracts were shaken with silicic acid and centrifuged. Cholesterol and triglycerides were determined in the supernatant using the Lieberman & Burchard reaction, and Van Handel & Zilversmit's (1957) method respectively. Blood glucose was determined according to Hugget & Nixon (1957).

Compounds. DMP was obtained via Fluka AG, Buchs, Switzerland; CMP was kindly synthesised by Dr. F. Rubessa, Department of Pharmaceutical Chemistry, University of Trieste. Other samples of these compounds were subsequently kindly supplied by Dr. G. C. Gerritsen, Upjohn Co., Kalamazoo, Michigan, U.S.A.

Results

The effect on plasma triglycerides of DMP and its metabolite CMP, given orally to fasted rats is shown in Table 1. A lowering of plasma triglycerides occurred with a dose of 1·5 mg/kg for both compounds. The effect increases with increase in dose up to about 50% inhibition; further increasing the dose after this point has been reached does not cause a significantly greater lowering of plasma triglycerides. Previous studies with DMP indicated that increasing the dose resulted in a prolongation of the lowering of plasma FFA rather than in an absolute increase in the fall of the plasma FFA (Bizzi & Garattini, 1966a).

Table 2 shows the time course of the activity of a single dose, 7·5 mg/kg orally, of DMP or its metabolite on FFA, triglycerides, cholesterol and phospholipids and also the level of blood glucose.

The most significant changes were at the expense of FFA and triglycerides, while phospholipids were slightly but not significantly lowered. The decrease in the concentration of plasma FFA always preceded the lowering of plasma triglycerides. The values of FFA were higher 6–8 hr after the treatment than after the controls. Plasma cholesterol and blood glucose were practically unchanged in these experimental conditions. The rate of decrease of plasma FFA and triglycerides when DMP and its metabolite were given intravenously was also investigated. Table 3 shows that the effect of the metabolite was rapid, a statistically significant decrease of plasma FFA occurring after 1 min. At 5 min there was a fall of about 50% while DMP decreased plasma FFA more slowly, showing a 50% decrease only after about 10 min. Plasma triglycerides changed only slightly during this experiment.

Discussion

The present results show that in fasted animals DMP and its metabolite, decrease not only FFA mobilization, but also the level of plasma triglycerides. The two drugs are similar in action orally, but show some differences when given intravenously. By the latter route the metabolite reduced plasma FFA in a shorter time than DMP. The fast rate at which

TABLE 1. EFFECT OF VARIOUS DOSES OF 3,5-DIMETHYLPYRAZOLE (DMP) OR 3-CARBOXY-5-METHYLPYRAZOLE (CMP) ON THE LEVELS OF PLASMA TRIGLYCERIDES

Treatment mg/kg oral	Triglycerides mg/100 ml ± s.e.	
	DMP	CMP
0	86 ± 5	86 ± 5
0·75	86 ± 4	80 ± 8
1·5	48 ± 1*	64 ± 4*
3·7	56 ± 1†	63 ± 4†
7·5	48 ± 3*	43 ± 3*
15	40 ± 2*	46 ± 4*
30	41 ± 2*	57 ± 4*

Rats fasted overnight; determination were made 4 hr after the treatment. Each figure represents the average of at least 5 determinations.
* P <0·01; † P < 0·05 relative to controls.

TABLE 2. EFFECT OF 3,5-DIMETHYLPYRAZOLE (DMP) OR 3-CARBOXY-5-METHYLPYRAZOLE (CMP) ON PLASMA LIPIDS AND BLOOD GLUCOSE

Time** min	FFA		Triglycerides		Phospholipids (phosphorus)		Cholesterol		Glucose	
	DMP	CMP	DMP	CMP	DMP	CMP	DMP	CMP	DMP	CMP
30	58*	58*	86	75	102	122	103	106	107	103
60	36*	29*	62*	76	85	83	86	88	103	94
120	42*	37*	76†	70†	93	80	90	—	102	120
240	69*	71*	53*	54*	88	85	96	91	86	106
360	143*	129	97	89	98	88	99	94	82	105
480	146	139*	124	87	97	88	108	100	68	98

** Time between dose and death; drugs were given orally in a single dose of 7·5 mg/kg; each figure represents the average of at least 5 determinations. Controls = 100. The absolute values were for FFA 633 ± 51 μ-equiv/litre; for triglycerides 74 ± 6 mg/100 ml; phosphorus in phospholipids 4·7 ± 0·3 mg/100 ml; cholesterol 71 ± 8 mg/100 ml; glucose 62 ± 7 mg%.
* P < 0·01; † P < 0·05 in respect to controls.

TABLE 3. LEVEL OF PLASMA FFA, TRIGLYCERIDES AND OF BLOOD GLUCOSE AFTER THE INTRAVENOUS ADMINISTRATION OF 3,5-DIMETHYLPYRAZOLE (DMP) OR 3-CARBOXY-5-METHYLPYRAZOLE (CMP)

Time after treatment (min)	FFA μ-equiv/litre ± s.e.			Triglycerides mg/100 ml ± s.e.		Glucose mg/100 ml ± s.e.	
	Control	CMP	DMP	Control	CMP	Controls	CMP
1	663 ± 22	509 ± 24*	715 ± 10	130 ± 8	130 ± 7	61 ± 5·7	70 ± 2·6
3	668 ± 35	507 ± 48*	732 ± 79	81 ± 4	108 ± 8	57 ± 4·1	67 ± 6·6
5	691 ± 49	299 ± 13*	554 ± 81*	87 ± 7	77 ± 1	66 ± 3·8	63 ± 3·2
10	594 ± 44	207 ± 21*	325 ± 7*	81 ± 2	68 ± 6	72 ± 6·6	72 ± 6·1
20	524 ± 41	146 ± 26*		91 ± 2	78 ± 5	70 ± 6·8	70 ± 4·6
30	529 ± 47	116 ± 21*		90 ± 2	69 ± 6†	70 ± 3·3	75 ± 4·6

Drugs were given i.v. at a dose of 3·7 mg/kg. Each figure is the average of at least 5 determinations.
P < 0·01; † P < 0·05 in respect to controls.

plasma FFA decrease after the metabolite was expected because of the known rapid turnover of FFA (Carlson & others, 1965).

The results obtained are also consistent with previous data showing that CMP but not DMP was effective in reducing lipolysis *in vitro* (Smith & others, 1965; Bizzi & Garattini, unpublished), and the findings agree with the hypothesis that the metabolite is the active compound responsible for the effect of DMP. It is interesting to note that the depression of plasma FFA was followed by a period of time (6–8 hr after treatment) in which a large increase of plasma FFA occurred. After the depression of plasma FFA there was always a decrease of plasma triglycerides probably related to a decrease of the liver triglyceride synthesis (Bizzi & Garattini, 1966b; Bizzi & others, 1966).

These results indicate that an important factor controlling the level of plasma and liver triglycerides in fasted rats may be the availability of plasma FFA. The level of plasma cholesterol and phospholipids was unaffected during the period in which FFA were depressed. Blood glucose was only slightly affected and the hypoglycaemia appeared to follow the decrease of FFA. These results are consistent with Randle's hypothesis that a depression of FFA should stimulate glucose utilization (Randle, Garland, Hales & Newsholme, 1963).

Since a high level of plasma triglycerides has been considered a possible negative factor in the development of atherosclerosis, thrombosis and coronary diseases (Albrink, 1960; Schrade & Boehle, 1960; Bizzi, Howard & Gresham, 1963) it is suggested that DMP and its metabolite, by lowering the levels of plasma FFA and triglycerides, might have some therapeutic value in the treatment of such diseases.

References

Alexander, W. B., MacDougall, A. I., Oliver, M. F. & Boyd, G. S. (1959). *Clin. Sci.*, **18**, 195–203.

Albrink, M. J. (1960). *Conn. Med.*, **24**, 27–30.

Altschul, R., Hoffer, A. & Stepnen, S. D. (1955). *Archs Biochem. Biophys.*, **54**, 558–559.

Bizzi, A. & Garattini, S. (1966a). *Methods in drug evaluation.* Editors: Mantegazza, P. & Piccinini, F., pp. 68–81. Amsterdam: North Holland Publishing Co.

Bizzi, A. & Garattini, S. (1966b). *2nd Symposium of drugs affecting Lipid Metabolism* New York: S. Karger.

Bizzi, A., Howard, A. N. & Gresham, A. G. (1963). *Nature, Lond.*, **19**, 195–196.

Bizzi, A., Jori, A., Veneroni, E. & Garattini, S. (1964). *Life Sci.*, **3**, 1371–1375.

Bizzi, A., Tacconi, M. T., Veneroni, E. & Garattini, S. (1966). *Nature, Lond.*, **209**, 1025–1026.

Carlson, L. A., Boberg, J. & Hogstedt, B. (1965). In *Handbook of Physiology, Sect.* 5. Editors: Renold, A. E. & Cahill, G. F. Pp. 625–644. Washington: American Physiological Society.

Deming, A. B., Hodes, M. E., Baltazar, A., Edreira, J. G. & Torosday, S. (1958). *Am. J. Med.*, **24**, 882–892.

Dole, V. P. (1956). *J. clin. Invest.*, **35**, 150–151.

Dury, A. & Treadwell, C. R. (1955). *J. clin. Endocr. Metab.*, **15**, 818–825.

Garattini, S. & Bizzi, A. (1966). *2nd Int. Symposium on Catecholamines, Pharmac. Rev.*, 1966, in the press.

Gerritsen, G. C. & Dulin, W. E. (1965a). *Diabetes*, **14**, 507–515.

Gerritsen, G. C. & Dulin, W. E. (1965b). *J. Pharmac. exp. Ther.*, **150**, 491–498.

Hollister, L. E., Kanter, S. L., Powell, P. B. & Henrich, U. L. (1957). *J. chron. Dis.*, **6**, 234–243.

Huggett, A. St. G. & Nixon, D. A. (1957). *Lancet*, **2**, 368–370.

Jacobs, R. S., Grebner, M. S. & Cook, D. L. (1965). *Proc. Soc. exp. Biol. Med.*, **119**, 1117–1120.

Lowry, O. H., Roberts, N. R., Leiner, K. Y. & Farr, M. L. (1954). *J. biol. Chem.*, **207**, 1–17.

Randle, P. J., Garland, P. B., Hales, C. N. & Newsholme, E. A. (1963). *Lancet*, **1**, 785–789.

Reid, J. (1961). *Drugs affecting Lipid Metabolism*, pp 423–431. Editors: Garattini, S. & Paoletti. R. Amsterdam: Elsevier Publishing Co.

Schrade, W. & Boehle, E. (1960). *Lancet*, **2**, 1409–1416.

Shafrir, E., Sussman, K. E. & Steinberg, D. (1959). *J. Lipid Res.*, **1**, 109–117.

Smith, D. L., Forist, A. A. & Dulin, W. E. (1965). *J. mednl Chem.*, **8**, 350–355.

Trout, D. L., Estes, E. H., Jr. & Friedberg, S. J. (1960). *J. Lipid Res.*, **1**, 199–202.

Van Handel, E. & Zilversmit, D. B. (1957). *J. Lab. clin. Med.*, **50**, 152–157.

Woods, K. R., Freedman, E. B. & Kellner, A. (1962). *Proc. Soc. exp. Biol. Med.*, **111**, 257–261.

Letters to the Editor

The effect of heparin on gastric secretion stimulated by histamine or ametazole hydrochloride.

SIR,—Recently it was found that parenterally administered degraded carrageenan markedly inhibits the gastric secretory response of the guinea-pig to histamine but not to 3-(2-aminoethyl)pyrazole dihydrochloride (ametazole hydrochloride; Histalog) (Watt, Eagleton & Marcus, 1966). It was of interest to determine whether the related sulphated polysaccharide, heparin, had a similar action.

Two groups of fasted adult male guinea-pigs were used. One group received heparin subcutaneously (400 mg/kg); the other group served as control. Nine hr after heparin administration both groups received an intramuscular injection of aqueous histamine acid phosphate (1 mg/kg); 1 hr later the gastric secretion was removed by intubation. Immediately thereafter the secretory test in the heparin-treated group was repeated using ametazole hydrochloride (100 mg/kg) instead of histamine. Results are presented in Table 1.

TABLE 1. EFFECT OF HEPARIN ON HISTAMINE- AND AMETAZOLE HYDROCHLORIDE-STIMULATED GASTRIC SECRETION (MEANS ± 1 s.d.)

Treatment and no. of animals	Gastric secretion: Volume (ml)	Gastric secretion: Total acid conc. (m-equiv/litre)
Group 1 Histamine only (10)	8·9 ± 2·2	128·6 ± 8·2
	$P < 0{\cdot}001$	$P < 0{\cdot}01$
Group 2 Heparin + histamine (8)	2·8 ± 1·4	110·9 ± 12·7
	$P < 0{\cdot}001$	$P > 0{\cdot}70$
Subsequent Ametazole HCl	11·9 ± 2·8	113·1 ± 23·1

Heparin markedly inhibits the gastric secretory response to histamine. Nevertheless, the stomach remains capable of responding well to ametazole hydrochloride. In spite of repeated intubation within so short a period, a factor which we have found to affect gastric secretion adversely, the volume of juice recovered in response to ametazole hydrochloride exceeded even that in the group of animals which had not received heparin.

This inhibitory effect of heparin on histamine-stimulated gastric secretion is similar to that previously noted in man and in the dog by Thompson, Lerner, Vakil & Tramontana (1963). Whether heparin works its effect by binding with histamine as these authors postulate, it is apparent that, as with degraded carrageenan, heparin does not block the acid secretory mechanism.

We thank Dr. E. A. G. Cook of Ely Lilly and Company, Basingstoke, for supplying Histalog.

Department of Pathology, University of Liverpool. — J. WATT, G. B. EAGLETON

Clatterbridge Hospital, Bebington, Cheshire. — R. MARCUS

June 21, 1966

References

Thompson, J. C., Lerner, H. J., Vakil, H. C. & Tramontana, J. A. (1933). *Surg. Forum*, **14**, 342-345.

Watt, J., Eagleton, G. B. & Marcus, R. (1966). *Nature, Lond.* In the press.

The effect of tricyclic antidepressive drugs on adrenaline and adenosine diphosphate induced platelet aggregation

SIR,—There is good agreement between the effects of adrenaline, noradrenaline and isoprenaline on the stimulation of the smooth muscle of the nictitating membrane, and on the aggregation of thrombocytes (O'Brien, 1964). Noradrenaline is weaker than adrenaline and isoprenaline has no effect at all. We have examined the effect of the tricyclic antidepressive drugs upon the aggregation of thrombocytes because these drugs inhibit the active uptake of adrenaline by the cells and thus may influence its action on the formation of microthrombi.

A second reason was the statement by Abood, Kimizuka, Rogeness & Biel (1963) that norimipramine probably interferes with the liberation of ATP from muscle cells in the course of depolarization. According to these authors norimipramine strengthens the cellular membrane and maintains the membrane potential.

If both adrenaline and ADP cause aggregation of thrombocytes in the same manner—by depolarizing the cellular membrane—then the tricyclic antidepressive drugs should have an inhibiting effect on the aggregation effect of both substances.

The antidepressive drugs of the tricyclic type were: imipramine, norimipramine, propazepine, amitriptyline and nortriptyline.

The experiments were made with a plasma rich in thrombocytes (Born & Cross, 1963). Using our modification of the method of O'Brien (1964) we found, in addition to the continuous fall in absorbance, another fall after 240 sec, following an additional 1 min of centrifuging at 25 rpm. During this procedure small flakes appeared, the dimensions of which excluded the possibility of plasma viscosity influencing their sedimentation. We also obtained more consistent results. The extent of the reduction, caused by the drugs, in the fall in absorbance with adrenaline or ADP is a measure of the inhibiting effects of the drugs. This is expressed as a percentage of the reduction in absorbance caused by adrenaline, 5×10^{-5}M, or ADP, 5×10^{-4}M, these concentrations being considered to give 100% aggregation.

The effects of the drugs on the aggregation of thrombocytes induced by adrenaline are shown in Table 1. This shows that the drugs at a concentration of 5×10^{-4}M completely block the aggregation of thrombocytes caused by adrenaline. A concentration of 5×10^{-5}M causes an inhibition of some 50%.

TABLE 1. INHIBITION OF THROMBOCYTES AGGREGATION BY TRICYCLIC ANTIDEPRESSIVE DRUGS. Aggregation induced by adrenaline in a concentration of 5×10^{-5} M

Inhibiting substance	conc. M	Inhibition %	s.d.	Number of experiments	Significance of inhibition P
Impramine	5×10^{-4}	100·62	7·16	6	<0·001
Imipramine	5×10^{-5}	45·64	35·56	15	<0·001
Norimipramine	5×10^{-4}	101·51	13·50	8	<0·001
Norimipramine	5×10^{-5}	52·92	7·71	8	<0·001
Amitriptyline	5×10^{-4}	101·39	10·83	4	<0·001
Amitriptyline	5×10^{-5}	41·69	29·22	10	<0·001
Nortriptyline	5×10^{-4}	104·41	4·91	10	<0·001
Nortriptyline	5×10^{-5}	65·88	28·68	11	<0·001

At 5×10^{-6}M the drugs have practically no effect. The differences between the effects of the individual substances are not statistically significant.

The effect of the drugs on the ADP-induced aggregation of thrombocytes is summarized in Table 2. Imipramine, amitriptyline and nortriptyline completely inhibit the aggregation caused by ADP when used in concentrations of 5×10^{-4}M. In concentrations of 5×10^{-5}M nortriptyline proved the most effective inhibitor. Concentrations of 5×10^{-6}M of all the drugs still caused an aggregation-inhibiting effect of some 20%.

TABLE 2. INHIBITION OF THROMBOCYTES AGGREGATION BY TRICYCLIC ANTIDEPRESSIVES. Aggregation induced by ADP

Inhibiting substance	Conc. M	Inhibition %	s.d.	Number of experiment	Significance of Inhibition P
Imipramine	5×10^{-4}	93·59	12·62	10	<0·001
Imipramine	5×10^{-5}	25·73	26·50	9	<0·05
Amitriptyline	5×10^{-4}	92·28	10·64	7	<0·001
Amitriptyline	5×10^{-6}	36·89	28·19	11	<0·001
Nortriptyline	5×10^{-4}	95·44	19·22	7	<0·001
Nortriptyline	5×10^{-6}	79·33	20·10	8	<0·001

It seems that the antidepressive drugs of the tricyclic type inhibit the effect of both adrenaline and ADP on the aggregation of thrombocytes in the same way. It may thus be assumed that both adrenaline and ADP have a direct effect on the cellular membrane of thrombocytes. Because of this, adrenaline does not intervene by the liberation of ADP. Imipramine probably strengthens the membrane of the thrombocytes and maintains their potential (Abood & others, 1963).

Medical Clinic of Postgraduate Medical Institute,
Prague-Krč,
Budějovická 800,
Czechoslovakia.
R. RYŠÁNEK

Research Institute for Experimental Therapy,
Prague-Krč,
Budějovická 800,
Czechoslovakia.
C. ŠVEHLA
H. ŠPÁNKOVÁ
M. MLEJNKOVÁ

June 6, 1966

References

Abood, L. G., Kimizuka, H. Rogeness, G. & Biel, J. H. (1963). *Ann.N.Y.Acad.Sci.*, **107**, 1139–1146.
Born, C. V. R. & Cross, M. J. (1963). *J.Physiol., Lond.*, **168**, 178–195.
O'Brien, J. R. (1964). *J.clin.Path.*, **17**, 275–281.

Programmed learning and long-term retention

SIR,—In a previous report (Foy, 1965) of an experiment using a programmed text for teaching physiology to first year undergraduate pharmacy students, it was concluded that students who read the programme were at no disadvantage either in learning time or amount learned, compared with those who followed a lecture on the same material. The results have since been confirmed with another group of students using an experimental programmed text on the control of respiration.

However, the results of examinations taken 4 months later suggested that students who had taken the programme enjoyed some advantage in the relevant sections of the papers over students in the lecture group—assuming that the two groups did not differ in the amount of explicit rehearsal of the material between the previous test and the examination. To test this suggestion, a second experiment was made. As in the first experiment (Foy, 1965), one group of students used a programmed text while the other group attended a lecture, the subject being "Control of Respiration". Also as before, certain experimental precautions (Cheris, 1964) were taken; the content to be learned was identical for each group, presentation was optimal, learning conditions were controlled and criterion testing was unbiased.

The first objective test was given 72 hr after the learning session. Students had notice of this test, and each group was asked not to collaborate in preparation with the other. A second, identical test was made after an interval of 7 weeks. For this test the students were given no notice, neither was any reference made to the investigation during the interval. The results of the two tests appear in Table 1. After the scores had been adjusted on the basis of a

TABLE 1. RETENTION OF MATERIAL LEARNED FROM LECTURES COMPARED WITH THAT FROM A PROGRAMMED TEXT

	Programme group	Lecture group
1. Students present at all 3 tests	32	32
2. Mark from previous objective test in physiology (60 possible): mean ± s.e.	29·7 ± 1·0	32·3 ± 1·0
3. Mark from test on respiratory control taken 72 hr after learning session (40 possible): mean ± s.e.	17·7 ± 0·9	19·7 ± 1·0
4. Mark from test on respiratory control taken 7 weeks after learning session (identical test); mean ± s.e.	13·9 ± 0·7	12·3 ± 1·0
5. Difference between 3 and 4	3·8 ± 0·9*	7·3 ± 0·9*
6. Number of students admitting to revision during retention interval	4	3

* Significant difference P = 0·99.

previous objective test in physiology (cf. item 2, Table 1) there was no significant difference between the performance of the two groups in the first test. However the "programme" group was significantly superior in the delayed retention test. As an approximate check on the assumption of no differential rehearsal between the groups, all the students were asked how much revision of the material they had done in the interval. As can be seen from Table 1 revision was minimal and balanced between the groups, so this does not seem to have been a significant factor. In fact, when the retention test data were re-examined after removal of the revising students' scores, there was no appreciable difference in the magnitude of the discrepancy between the two groups.

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School of Research in Education,
School of Pharmacy,
Bradford Institute of Technology,
Bradford 7.
June 8, 1966

B. Cooper
J. M. Foy

References

Cheris, B. H. (1964). *J. med. Educ.*, **39**, 304–310.
Foy, J. M. (1965). *J. Pharm. Pharmac.*, **17**, 385–386.

Importance of noradrenaline synthesis for the interaction between desipramine and reserpine

Sir,—We have previously reported that the hyperthermic effect induced by desipramine in rats given reserpine may be inhibited by peripheral adrenergic blocking agents (Jori, Paglialunga & Garattini, 1966) and by α-methyl-*m*-tyrosine (Garattini & Valzelli, 1961), an agent which depletes brain noradrenaline stores (Hess, Connmacher, Ozaki & Udenfriend 1961; Gessa, Costa, Kuntzman & Brodie, 1962).

These results suggested that imipramine-like drugs might antagonise the reserpine hypothermia by interacting with the adrenergic system. In fact, an increase of the concentration of noradrenaline at the receptor sites might be expected as a consequence of the inhibitory action of desipramine on the catecholamine re-uptake at the nerve endings (Iversen, 1965).

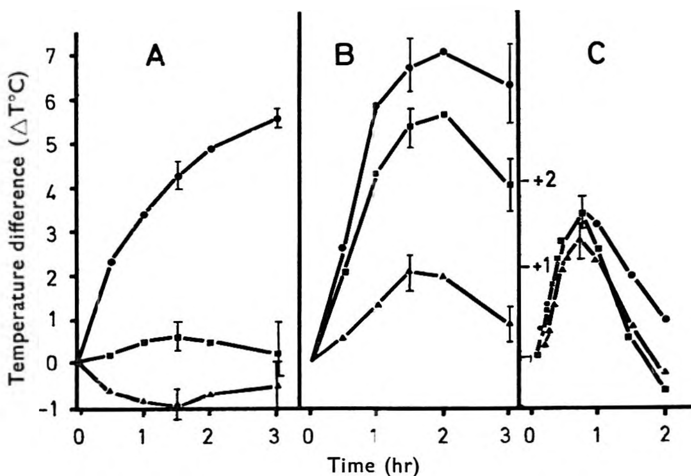

Fig. 1. Hyperthermic agents (desipramine, dopa and noradrenaline) were given at zero time, 18 hr after reserpine and 2 hr after the enzymatic inhibitors (α-methyl-*p*-tyrosine and diethyldithiocarbamate). Each point represents the average of 8 rats. The vertical bars indicate the standard errors. A. ●——● desipramine (7·5 mg/kg i.p.). ■——■ α-Methyl-*p*-tyrosine (80 mg/kg i.p.) + desipramine. ▲——▲ Diethyldithiocarbamate (300 mg/kg i.p.) + desipramine. B. ●——● Dopa (150 mg/kg i.p.). ■——■ α-Methyl-*p*-tyrosine + dopa. ▲——▲ Diethyldithiocarbamate + dopa. C. ●——● Noradrenaline (300 μg/kg i.v. in 15 min.). ■——■ α-Methyl-*p*-tyrosine + noradrenaline. ▲——▲ Diethyldithiocarbamate + noradrenaline.

In an attempt to adduce further evidence in support of this hypothesis, some experiments were made to establish if the inhibition of noradrenaline biosynthesis effected a decrease in desipramine-induced hyperthermia in reserpinized rats. Noradrenaline synthesis was blocked with either α-methyl-*p*-tyrosine —an inhibitor of tyrosine hydroxylase (Spector, Sjoerdsma & Udenfriend, 1965; Torchiana, Stone & Porter, 1965)—or diethyldithiocarbamate—an inhibitor of dopamine-β-hydroxylase (Collins, 1965; Carlsson, Lindqvist, Fuxe & Hökfelt, 1966).

Female Sprague-Dawley rats were given reserpine (5 mg/kg i.v.) and 16 hr after either α-methyl-*p*-tyrosine (80 mg/kg) or diethyldithiocarbamate (300 mg/kg) was injected intraperitoneally. Desipramine (7·5 mg/kg i.p.) was given 2 hr after the inhibitors. In other experiments dopa (150 mg/kg i.p.) or noradrenaline (45 μg/rat infused in 15 min) was given instead of desipramine.

When the hyperthermic agents were injected, rats were placed in individual cages and temperatures recorded with an automatic device (Jori & Paglialunga, 1966).

All the experiments were made at an environmental temperature of 20° with a relative humidity of 56%.

Fig. 1 shows that desipramine, dopa and noradrenaline increase, although to a different extent, the body temperature in fully reserpinized rats. The hyperthermia induced by desipramine, but not that induced by noradrenaline, is blocked by α-methyl-*p*-tyrosine and by diethyldithiocarbamate Dopa-induced hyperthermia is blocked by diethyldithiocarbamate but not by α-methyl-*p*-tyrosine. These findings are consistent with the results that should be expected considering the postulated site of enzymatic inhibition exerted by α-methyl-*p*-tyrosine and by diethyldithiocarbamate. We therefore conclude that the presence of noradrenaline itself is essential for the achievement of desipramine-induced hyperthermia in reserpinized animals.

Instituto di Ricerche Farmacologiche 'Mario Negri',
Via Eritrea, 62,
Milan,
Italy.

A. JORI
M. C. CARRARA
S. GARATTINI

June 8, 1966

References

Carlsson, A., Lindqvist, M., Fuxe, K. & Hökfelt, T. (1966). *J. Pharm. Pharmac.*, **18**, 60–62.
Collins, G. G. S. (1965). *Ibid.*, **17**, 526–527.
Garattini, S. & Valzelli, L. (1961). In *Monoamines et Systéme Nerveux Centrale*, Bel-air Symposium, Geneva, pp. 59–88.
Gessa, G. L., Costa, E., Kuntzman, R. & Brodie, B. B. (1962). *Life Sci.*, **8**, 353–360.
Hess, S. M., Connmacher, R. H., Ozaki, M. & Udenfriend, S. (1961). *J. Pharmac. exp. Ther.*, **134**, 129–138.
Iversen, L. L. (1965). *J. Pharm. Pharmac.*, **17**, 62–64.
Jori, A., Paglialunga, S. & Garattini, S. (1966). *Ibid.*, **18**, 326–327.
Jori, A. & Paglialunga, S. (1966). *Med. Pharmac. exp.*, in the press.
Spector, S., Sjoerdsma, A. & Udenfriend, S. (1965). *J. Pharmac. exp. Ther.*, **147**, 86–95.
Torchiana, M. L., Stone, C. A. & Porter, C. C. (1965). *Fedn. Proc. Fedn Am. Socs exp. Biol.*, **24**, 735.

The intracerebral effects of noradrenaline and its modification by drugs in the mouse

SIR,—Imipramine-like antidepressant drugs are known to potentiate the peripheral pharmacological effects of noradrenaline (Osborne & Sigg, 1960: Cairncross, 1965; Jori & Garattini, 1965). The mechanism has been attributed to their ability to inhibit the uptake of the exogenous catecholamine (Axelrod, Whitby & Hertting, 1961; Iversen, 1965) and a similar mechanism has been proposed for imipramine-like drugs in the central nervous system (Glowinski & Axelrod, 1964). However, there is little direct evidence about the effects of imipramine and related drugs on the pharmacological effects induced by noradrenaline administered intracerebrally. The effects of noradrenaline injected intracerebrally in the conscious mouse and their modification by oral pretreatment with imipramine-like antidepressants and other pharmacological agents are now reported.

Noradrenaline was injected intracerebrally (Haley & McCormick, 1957) in male albino mice (Glaxo A_2G strain). The site of injection was within 1 mm of a point on the midline 2 mm rostral to a line joining the anterior bases of the ears. The injection was made with a 22 gauge needle $\frac{1}{8}$ inch long attached to a 0·25 ml Bacton, Dickinson & Co. tuberculin syringe inserted perpendicularly through the skull and into the brain, in volumes of 0·02 ml per mouse. The site was checked by injecting a 1 in 10 dilution of Indian ink in 0·9% sodium chloride solution. Histological examinations reveal particles of ink in the third and fourth ventricles and occasionally along the injection route. Oesophageal temperatures were measured with an electric thermometer and thermocouple (Brittain & Spencer, 1964). In drug interaction experiments the test compounds were orally administered to groups of 5 mice 1 hr before the intracerebral injection of noradrenaline (Acute test). For some drugs, three doses were administered 24, 16 and 1 hr before injection of noradrenaline intracerebrally (Subacute test).

After the intracerebral injection of saline animals remained quiet for 1–2 min before resuming their normal activity when they did not differ from untreated animals. Although a slight and transient fall in body temperature did occur,

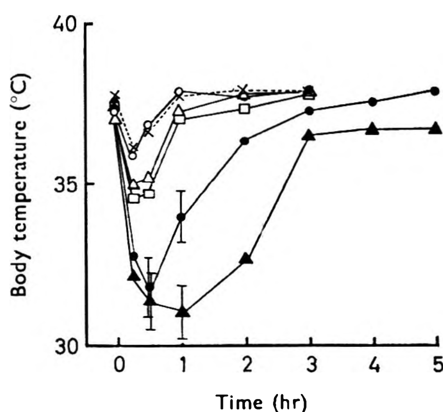

FIG. 1. Changes in body temperature following the intracerebral injection of noradrenaline. Doses μg/mouse: 1 (—○—), 2·5 (—△—), 5 (—□—), 10 (—●—) and 20 (—▲—). Saline control (--X--).

no animal showed residual detrimental effects from the intracerebral injection of saline. In contrast, the intracerebral injection of 10 μg of noradrenaline caused motor inco-ordination and ataxia and after 30 min the animals were sedated and could not be aroused. It was found that the sedation was accompanied by a fall in body temperature. The effect of graded intracerebral doses of noradrenaline on body temperature are shown in Fig. 1.

Pretreatment of animals with imipramine, amitriptyline or nortripytline antagonised the depressive and hypothermic actions of noradrenaline. The effects on body temperature in acute and subacute tests are shown in Fig. 2A and B respectively. The antagonistic effect of imipramine is dose dependent especially in the initial hyperthermia which occurs after noradrenaline injection in pretreated animals.

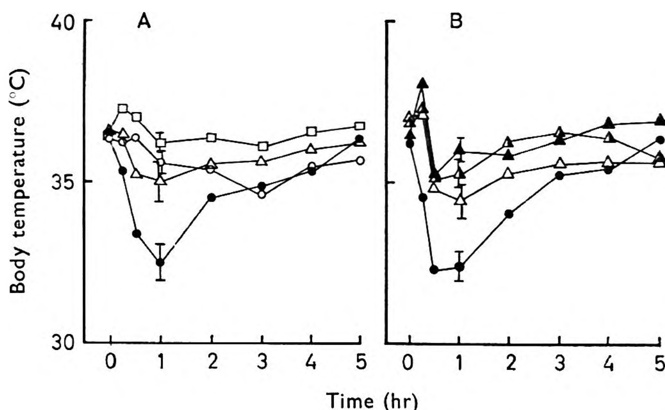

FIG. 2. Effects of imipramine-like antidepressants on hypothermia induced by intracerebrally injected noradrenaline (10 μg/mouse). A. Imipramine (—△—), amitriptyline (—○—) and nortriptyline (—□—) each at 5 mg/kg orally 1 hr prior to noradrenaline. Noradrenaline control (—●—). B. Imipramine, 3 oral doses each at 5 mg/kg (—△—), 10 mg/kg (—▲—) or 20 mg/kg (—▲—) before noradrenaline. Noradrenaline control (—●—).

The effects of other drugs have been investigated in this procedure. The drugs listed below did not prevent noradrenaline-induced hypothermia nor depression in doses (mg/kg orally) up to those given in brackets after each compound: chlorpromazine (2), chlordiazepoxide (25), diazepam (25), haloperidol (25), pentobarbitone (25), phenytoin (25), benzhexol (10), atropine (10), chlorpheniramine (10) and homochlorcyclazine (10). Higher doses of atropine (20) and benzhexol (20) were weakly active in preventing the noradrenaline-induced hypothermia by about 50%. Amphetamine (2) almost completely prevented the intracerebral effects of noradrenaline. Phenelzine (5) had little effect on the noradrenaline response but sub-acute administration of this drug (3 × 5 mg) potentiated the hypothermia and sedation.

The mode of action of the imipramine-like antidepressants in antagonising the central effects of noradrenaline is not known. Preliminary experiments in which uptake of tritrated noradrenaline has been studied indicate that very small amounts (1·8–2·8%) of the injected noradrenaline are taken up by the

brain tissue. Furthermore imipramine, in doses which antagonized the depression and hypothermia produced by noradrenaline, had no significant effect on this very low uptake. Thus it is impossible at this time to reconcile the observed antagonism of noradrenaline by imipramine-like antidepressants in the mouse with the current concept that these agents may facilitate central adrenergic mechanisms in the same way as they do at the periphery by inhibiting the uptake of noradrenaline into neurone transmitter stores (Axelrod & others, 1961; Iversen, 1965; Jori, Paglialunga & Garattini, 1966).

Research Division,
Allen & Hanbury Ltd.,
Ware, Herts.

R. T. Brittain

May 20, 1966

References

Axelrod, J., Whitby, L. G. & Hertting, G. (1961). *Science, N.Y.*, **133**, 383–384.
Brittain, R. T. & Spencer, P. S. J. (1964). *J. Pharm. Pharmac.*, **16**, 497–499.
Cairncross, K. D. (1965). *Archs int. Pharmacodyn. Thér.*, **154**, 438–448.
Glowinski, J. & Axelrod, J. (1964). *Nature, Lond.*, **204**, 1318–1319.
Haley, T. J. & McCormick, W. G. (1957). *Br. J. Pharmac. Chemother.*, **12**, 12–15.
Iversen, L. L. (1965). *J. Pharm. Pharmac.*, **17**, 62–64.
Jori, A. & Garattini, S. (1965). *Ibid.*, **17**, 480–488.
Jori, A., Paglialunga, S. & Garattini, S. (1966). *Ibid.*, **18**, 326–327.
Osborne, M. & Sigg, E. B. (1960). *Archs int. Pharmacodyn. Thér.*, **129**, 273–288.

On the mechanism of the hyperglycaemic effect of chlorpromazine

Sir,—Chlorpromazine shows hyperglycaemic properties in several animal species including man. Previous experiments suggested that this drug interferes with the peripheral utilization of glucose (Chagovets & Shtutman, 1963; Jori, Bernardi & Garattini, 1964) as supported by the fact that chlorpromazine reduces glucose tolerance (Bhide, Tiwari & Balwani, 1965; Jori & Bianchetti, 1966). However, several mechanisms may be involved (Bonaccorsi, Jori & Garattini, 1964). In high doses, chlorpromazine may induce hyperglycaemia by activating adrenals and sympathetic nerve endings (Mraz & Triner, 1963).

Chlorpromazine also released adrenaline from adrenals *in vitro* (Weil-Malherbe & Posner, 1963) and it increased the urinary excretion of catecholamines *in vivo* (Johnson, 1964). Furthermore chlorpromazine impaired the uptake

TABLE 1. EFFECT OF β-ADRENERGIC BLOCKING AGENTS ON THE HYPERGLYCAEMIA INDUCED BY CHLORPROMAZINE AND BY ADRENALINE

Treatment mg/kg i.p.	Blood glucose (mg %) after chlorpromazine 15 mg/kg	Blood glucose (mg %) after adrenaline 100 μg/kg
Saline	137 ± 9	146 ± 3
MJ 1999 5 × 2	102 ± 2·6*	109 ± 2·4*
Propanolol 5 × 2	99 ± 5*	96 ± 4*
Pronethalol 5 × 2	116 ± 6·1*	112 ± 7·4
D-(−)-INPEA 10	108 ± 2*	106 ± 5*

Blood Glucose was measured 1 and 2 hr after adrenaline and chlorpromazine respectively.

* P $<0·01$ for saline treatment.

Untreated animals show a blood glucose level of 70 ± 5 mg %. β-Blocking drugs were given 1 hr before and 15 min after the administration of chlorpromazine or adrenaline. D-(−)-INPEA was given only 1 hr before the hyperglycaemic agent.

of catecholamines (Hertting, Axelrod & Whitby, 1961) making available more amine for the receptor sites.

This action may produce a different effect according to the receptor site involved (Thoenen, Hürlimann & Haefely, 1965). Chlorpromazine blocks the α- but not the β- adrenergic receptors. It may be that the hyperglycaemic effect of chlorpromazine, looked upon as a β-receptor effect, follows from the release of adrenaline. The following experiments support this view.

Sprague-Dawley rats, fasted for 16 hr, received a combination of non-hyperglycaemic doses of chlorpromazine (5 mg/kg i.p.) and 6 hr later an infusion of adrenaline (1·5 μg/kg) for 20 min. The resulting hyperglycaemia was more pronounced and longer lasting with the combination than for each drug. For example the concentration of blood glucose 10 min after the end of infusion was 154 mg % in chlorpromazine pretreated rats and 116 mg % with rats given only adrenaline.

In other experiments the effect of several β-adrenergic blocking agents was challenged on the hyperglycaemia obtained in 18 hr fasted rats by injection of 15 mg/kg i.p. of chlorpromazine or of 100 μg/kg s.c. of adrenaline. Blood glucose levels were measured by an enzymatic method (Hugget & Nixon, 1957).

The results in Table 1 show that the various β-blocking agents can prevent the hyperglycaemia induced either by chlorpromazine or adrenaline in fasted animals.

Acknowledgment. MJ 1999(4-(2-isopropylamino-1-hydroxyethyl)methanosulphonanilide) was kindly supplied by Mead Johnson, Evansville, Indiana; propranolol and pronethalol by I.C.I., London and D-(−)-INPEA (*N*-isopropyl-*p*-nitrophenylethanolamine) by Laboratorio Bioterapico Selvi, Milan.

Instituto di Recerche Farmacologiche 'Mario Negri',
Via Eritrea 62,
Milan,
Italy.
June 8, 1966

A. Jori
M. C. Carrara

References

Bonaccorsi, A., Jori, A. & Garattini, S. (1964). *Br. J. Pharmac., Chemopher.*, **23**, 93–100.
Bhide, M. B., Tiwari, N. M. & Balwani, J. H. (1965). *Archs int. Pharmacodyn. Thér.*, **156**, 166–171.
Chagovets, R. V. & Shtutman, M. (1963). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **22**, T 935–937.
Hertting, G., Axelrod, J. & Whitby, L. G. (1961). *J. Pharmac. exp. Ther.*, **134**, 146–153.
Hugget, A. & Nixon, D. A. (1957). *Biochem. J.*, 66, 12P.
Johnson, G. E. (1964). *Acta physiol. scand.*, **60**, 181–188.
Jori, A., Bernardi, S. & Garattini, S. (1964). *Int. J. Neuropharmac.*, **3**, 553–558.
Jori, A. & Bianchetti, A. (1966). *Ibid.*, in the press.
Mraz, M. & Triner, L. (1963). *Archs int. Pharmacodyn. Thér.*, **141**, 434–442.
Thoenen, H., Hürlimann, & Haefely, W. (1965). *Int. J. Neuropharmac.*, **4**, 79–89.
Weil-Malherbe, H. & Posner, H. S. (1963). *J. Pharmac. exp. Ther.*, **140**, 93–102.

Inhibition of the taenia of the guinea-pig caecum by acetylcholine, nicotine or 5-hydroxytryptamine

SIR,—During an analysis of the mechanisms of the contractile responses of the taenia induced by drugs, it was observed that in the presence of hyoscine (0·1 μg/ml), nicotine (1–10 μg/ml) produced a relaxation of the preparation. High doses of acetylcholine (10–20 μg/ml) or 5-hydroxytryptamine (5-HT) (10–20 μg/ml) induced a biphasic effect—a relaxation followed by a contraction. The size of these inhibitory responses varied greatly and was dependent on the tone of the preparation, and this tone often varied in the course of an experiment. No inhibitory responses were observed on preparations which did not exhibit tone. It was found that a preparation with a stable degree of tone could be obtained with histamine (0·2 μg/ml) included in the bath fluid. This concentration of histamine produced more than 80% of the maximal contraction of the taenia. In the presence of this artificial tone, inhibitory responses were recorded with nicotine (2–20 μg/ml), acetylcholine (20–100 μg/ml) and 5-HT (10–100 μg/ml). The mechanisms of these responses were investigated.

A 5 cm length of the taenia was set up in Krebs solution at 37° containing hyoscine (0·1 μg/ml) and histamine (0·2 μg/ml). The solution was gassed with a mixture of oxygen 95% and carbon dioxide 5%. The concentrations of the drugs are expressed as the base. The responses were recorded on a smoked drum by means of an isotonic side-writing balsa wood lever.

The inhibitory responses to nicotine or acetylcholine were blocked by hexamethonium (20–40 μg/ml), dimethylphenylpiperazinium (5 μg/ml) or pentolinium (5 μg/ml). The local anaesthetic agents, procaine (10 μg/ml) or cocaine (1–20 μg/ml), abolished the relaxations caused by acetylcholine and greatly reduced those produced by nicotine. Higher concentrations of these drugs reduced the artificially induced tone and thus made the interpretation of their effects on the responses difficult. However, when experiments were made with preparations which exhibited tone in the absence of histamine, 50 μg/ml of procaine blocked the relaxations to nicotine or to acetylcholine. The inhibitory responses to 5-HT were not modified by any of these agents.

Guanethidine (1–10 μg/ml) reduced the responses to nicotine, blocked those to acetylcholine, but did not antagonize the relaxation produced by 5-HT. The responses to acetylcholine or to nicotine returned as soon as the blocking drug was washed out of the bath. This is in contrast to the usual persistent adrenergic blockage produced by this compound (Maxwell, Plummer, Schneider, Povalski & Daniel, 1960).

It was found that noradrenaline or isoprenaline relaxed the taenia. The responses to isoprenaline, but not to noradrenaline, were eliminated by pronethalol (1–2 μg/ml). The responses to nicotine or acetylcholine were reduced by this β-adrenergic blocking drug, but the effect of 5-HT remained unchanged. Hydergine (a mixture of equal parts of dihydroergocornine, dihydroergocryptine and dihydroergocristine) at a concentration of 3 μg/ml almost abolished the inhibitory responses to noradrenaline but not those produced by isoprenaline. The inhibition induced by acetylcholine or nicotine but not that caused by 5-HT was reduced by hydergine. Phenoxybenzamine (1 μg/ml) or piperoxan (1–5 μg/ml) antagonized the histamine-induced tone.

Two guinea-pigs were pretreated with reserpine (10 mg/kg) 24 hr before the experiment and two others were given reserpine 0·5 mg/kg daily for 10 days. A daily dose of 15 mg/kg of guanethidine was given to two guinea-pigs for 3 days and the animals were killed on the fourth day. In all the experiments made with preparations from these animals there was no evidence that the pretreatment modified the responses.

These experiments may be summarized in Table 1.

TABLE 1. SUMMARY OF THE EFFECTS OF ANTAGONISTS ON THE INHIBITORY RESPONSES

Antagonist	Relaxation to:		
	Acetylcholine	Nicotine	5-Hydroxytryptamine
Ganglion blocking agent (hexamethonium or pentolinium)	blocked	blocked	no effect
Procaine or cocaine	blocked	blocked	no effect
Guanethidine	blocked (non-persistent) effect	Reduced (non-persistent) effect	no effect
Pronethalol or Hydergine	reduced	reduced	no effect
Pretreatment with reserpine or guanethidine	no effect	no effect	no effect

Weis (1962) reported that dimethylphenylpiperazinium relaxed the isolated taenia of the guinea-pig caecum, and attributed the response to either the stimulation of adrenergic nerves or to the release of catecholamines from chromaffin cells.

The result I now report with ganglion blocking drugs or local anaesthetic agents are consistent with the involvement of a nerve pathway in the responses to nicotine or acetylcholine. But the evidence is not wholly consistent with the view that these drugs stimulated adrenergic nerves. Thus, although guanethidine reduced the responses, the characteristic prolonged blockade (Maxwell & others, 1960) was not seen. In fact, the blocking effect resembled the ganglion blocking activity of guanethidine (Maxwell & others, 1960).

Reserpine or guanethidine pretreatment did not seem to modify the relaxations to nicotine of acetylcholine. Similar concentrations of guanethidine (Boyd, Gillespie & MacKenna, 1962) or reserpine (Bentley, 1962; Day & Rand, 1964) have been shown to reduce or abolish the responses of other intestinal preparations to sympathetic nerve stimulation. The result of these pretreatments also renders it unlikely that these drugs induced the responses by the release of catecholamines from (non-innervated) chromaffin cells.

The most acceptable interpretation of these experiments appears to be that nicotine or acetylcholine stimulated inhibitory nerves which did not release noradrenaline (or adrenaline) at their terminations. Burnstock, Campbell & Rand (1966) have also reached a similar conclusion that there may be inhibitory nerves distinct from adrenergic nerves.

The relaxations of the taenia produced by 5-HT were not modified by any of the antagonists used. It seems probable that this effect of 5-HT arose from a direct action on the smooth muscle cells.

Department of Pharmacology,
King's College,
Strand,
London, W.C.2.

P. I. AKUBUE

June 20, 1966

Present address:
Department of Veterinary Anatomy and Physiology,
University of Ibadan, Nigeria.

References

Bentley, G. A. (1962). *Br. J. Pharmac. Chemother.*, **19**, 85–98.
Boyd, H., Gillespie, J. S. & MacKenna, B. R. (1962), *Ibid.*, **19**, 258–270.
Burnstock, G., Campbell, G., & Rand, M. J. (1966). *J. Physiol., Lond.*, **182**, 504–526.
Day, M. D. & Rand, M. J. (1964). *Br. J. Pharmac. Chemother.*, **22**, 72–86.
Maxwell, R. A., Plummer, A. J., Schneider, F., Povalski, H. & Daniel, A. I. (1960). *J. Pharmac. exp. Ther.*, **128**, 22–29.
Weis, J. (1962). *Acta pharmac. tox.*, **19**, 121–128.

The role of dopamine in motor excitation of mice induced by brain catecholamine releasers

SIR,—Animals treated with monoamine oxidase inhibitors show intense behavioural excitation after the administration of drugs that release catecholamines in the brain. This excitation is currently interpreted as the effect of an excess of free and active amines reaching their receptors (Brodie, Pletscher & Shaw, 1956; Costa, Gessa, Kuntzman & Brodie, 1962; van Rossum & Hurkmans, 1963; Graeff, Garcia Leme & Rocha e Silva, 1965). However the specific role played by noradrenaline or dopamine in this phenomenon is still uncertain.

The experiments now presented suggest a predominant participation of dopamine in the psychomotor stimulation of mice after reserpine or α-methyl-*m*-tyrosine when given after a monoamine oxidase inhibitor.

Seventy male albino mice, 20–25 g, were divided into 7 groups for different drug treatments. In each experiment one pair of mice had its motor activity continuously registered during the 5 hr after the last drug injection by means of a photoelectric actometer (van Rossum & others, 1962). The treatment schedule and the results are summarized in Table 1. All drugs were dissolved in saline except reserpine (Serpasol, Ciba, Brazil) which was diluted in distilled water.

TABLE 1. INHIBITION BY α-METHYL-*m*-TYROSINE OF MOTOR STIMULATION CAUSED BY BRAIN CATECHOLAMINE RELEASE IN MICE

	Treatment (doses in mg/kg)	Activity counts*
I	Saline (i.p.) 15 min after MAOI†	3·92 ± 2·81
II	Reserpine (10, i.p.) 15 min after MAOI	60·26 ± 7·41
III	Methyltyrosine (160, i.v.) and after 5 hr, MAOI + reserpine (10, i.p.)	10·44 ± 7·14
IV	Methyltyrosine (160, i.v.) and after 24 hr, MAOI + reserpine (10, i.p.)	57·50 ± 3·71
V	Methyltyrosine (50, i.p. 15 min. after MAOI	38·98 ± 8·93
VI	Methyltyrosine (160, i.v.) and after 5 hr, MAOI — methyltyrosine (50, i.p.)	11·76 ± 4·78
VII	Methyltyrosine (160, i.v.) and after 24 hr. MAOI + methyltyrosine (50, i.p.)	36·72 ± 11·21

*Total number of impulses recorded during 100 min of maximal activity; figures represent the mean and standard error of 5 pairs of mice.
† Monoamine oxidase inhibitor: *N*-(1,4-Benzodioxan-2-yl)-*N*-benzylhydrazine tartrate (2596-IS, base-62%), 80 mg/kg i.p.

Five hr after the depleting dose of α-methyl-*m*-tyrosine (160 mg/kg, i.v.) there was a sharp reduction in the psychomotor stimulation induced by reserpine or methyltyrosine (50 mg/kg, i.p.) injected after monoamine oxidase inhibition; the response returned to control values 24 hr later. Data reported by Costa & others (1962) indicate that the dose of methyltyrosine employed (160 mg/kg, i.v.) gives an almost complete depletion of brain noradrenaline of several days' duration whilst dopamine is only transitorily decreased; the maximum dopamine depletion occurs around 4 hr after the injection and the normal concentration is almost restored 24 hr later.

Our results suggest that a normal dopamine store is the only requirement for the production of motor stimulation by catecholamine releasers; however, the

possibility of dopamine interacting with noradrenaline receptors in the brain (Carlsson, 1966) remains.

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Department of Pharmacology, F. G. GRAEFF
Faculty of Medicine,
Ribeirão Preto, S.P.,
Brazil.
June 30, 1966.

References

Brodie, B. B., Pletscher, A. & Shore, P. A. (1956). *J. Pharmac. exp. Ther.*, **116**, 9.
Carlsson, A. (1966). In *5-Hydroxytryptamine and Related Indolealkylamines*, pp. 529–592. Berlin: Springer-Verlag.
Costa, E., Gessa, G. L., Kuntzman, R. & Brodie, B. B. (1962). In *First Int. Pharmacol. Meeting*, vol. 8, pp. 43–71, Oxford: Pergamon Press.
Graeff, F. G., Garcia Leme, J. & Rocha e Silva, M. (1965). *Int. J. Neuropharmac.*, **4**, 17–26.
van Rossum, J. M. & Hurkmans, J. A. Th. M. (1963). *J. Pharm. Pharmac.*, **15**, 493–499.
van Rossum, J. M., van Ameron, W. F. H., Daamen, J. L., Hurkmans, J. A. Th.M., Megens, G. J. J. & Peters, K. A. (1962). *Experientia*, **18**, 93–96.

On the mechanism of chlorpromazine-induced changes of cerebral homovanillic acid levels

SIR,—In various animal species, chlorpromazine and other neuroleptic drugs increase the concentration of the dopamine metabolite 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid) in the brain, especially in the extrapyramidal centres, without markedly interfering with the content of dopamine and 5-hydroxyindoleacetic acid (Andén, Roos & Werdinius, 1964; Gey & Pletscher, 1964; Juorio, Sharman & Trajkov 1966; Laverty & Sharman, 1965; Da Prada & Pletscher, 1966; Roos, 1965). Hydroxylation of tyrosine is thereby enhanced (Burkard, Gey & Pletscher, 1966). The question has been raised whether neuroleptics might enhance the formation of dopamine through a feedback mechanism due to blockade of dopaminergic receptors (Carlsson & Lindqvist, 1963; Gey & Pletscher, 1964; Da Prada & Pletscher, 1966). The results of the present experiments accord with this assumption and indicate that the storage sites of dopamine are possibly involved in the feedback mechanism.

Normal rats were injected i.p. with various psychotropic drugs (see Table 1). In addition, 10 mg/kg chlorpromazine was administered subcutaneously at various time intervals after intraperitoneal injection of 2·5 mg/kg reserpine. The animals were kept at an environmental temperature of 31–32° so that the rectal temperature remained normal within a range of ±1–2° during the course of the experiments. Homovanillic acid, 5-hydroxyindoleacetic acid (5-HIAA) and dopamine were measured in the brain stem (including basal ganglia, but without medulla oblongata and pons) with spectrophotofluorimetric methods (Andén, Roos & Werdinius, 1963; Carlsson & Waldeck, 1958; Pletscher, Burkard & Gey, 1964).

Neuroleptics of various chemical structures (chlorpromazine, chlorprothixene, haloperidol), in contrast to thymoleptics (imipramine, amitriptyline), tranquillisers (meprobamate, chlordiazepoxide, diazepam), and hypnotics (pheno-

barbitone, hexobarbitone), markedly increased the homovanillic acid content of the brain stem. Whereas none of the drugs produced more than a moderate elevation of the level of 5-HIAA, the thymoleptics even caused a slight but significant decrease of this substance (Table 1).

These findings suggest the possibility of a relationship between disturbed extrapyramidal function and increased formation of cerebral homovanillic acid. Thus, only those psychotropic drugs, i.e. the neuroleptics, which are known to interfere markedly with the function of the extrapyramidal centres in man and animals cause a major increase of the cerebral homovanillic acid levels. Since

TABLE 1.—THE EFFECTS OF PSYCHOTROPIC DRUGS ON THE CONTENT OF HOMOVANILLIC ACID (HVA) AND 5-HYDROXYINDOLEACETIC ACID (5-HIAA) IN THE BRAIN STEM OF RATS

Drug	Dose	HVA	Dose	5-HIAA
Chlorpromazine	10	314 ± 21	20	119 ± 3
Chlorprothixene	10	300 ± 17	20	125 ± 4
Haloperidol	5	340 ± 8	10	122 ± 5
Imipramine	10	91 ± 9	20	72 ± 3
Amitriptyline	10	125 ± 17	20	82 ± 5
Meprobamate	50	115 ± 8	50	130 ± 6
Chlordiazepoxide	50	83 ± 5	50	132 ± 7
Diazepam	10	83 ± 8	10	124 ± 5
Phenobarbitone	50	100 ± 17	50	111 ± 5
Hexobarbitone	50	100 ± 17	50	120 ± 14

The results are expressed in percent of untreated controls and represent averages of 4–10 experiments ± s.e. The drugs were administered i.p. 2–3 hr before death.

at least part of the extrapyramidal system seems to be dopaminergic (Sourkes, 1961; Andén, Carlsson, Dahlström & others, 1964; Carlsson, 1964; Hornykiewicz, 1964), a blockade of dopaminergic receptors by the neuroleptics and a secondary enhancement of dopamine synthesis, for example by a feedback mechanism, is conceivable.

Experiments with reserpine plus chlorpromazine indicate that the storage sites for dopamine may be involved in the feedback mechanism. Thus, after depletion of the dopamine stores by reserpine, chlorpromazine no longer causes a major rise in homovanillic acid, although reserpine does not seem markedly to decrease the activity of the enzymes involved in dopamine biosynthesis (Glowinski, Iverson & Axelrod, 1966). Both the decrease of the dopamine content and the inhibition of the chlorpromazine-induced rise in homovanillic acid follow a similar time course (Table 2).

TABLE 2. THE EFFECT OF RESERPINE ON THE CHLORPROMAZINE-INDUCED INCREASE OF HOMOVANILLIC ACID (HVA) AND ON THE DOPAMINE CONTENT IN THE BRAIN STEM OF RATS

Hr after reserpine	HVA*	Dopamine†
0 (controls)	100 ± 15	100 ± 3
2	—	20 ± 4
4	8 ± 3	—
7	—	19 ± 4
19	19 ± 6	20 ± 4
50	35 ± 17	36 ± 2

In the experiments with HVA, 10 mg/kg chlorpromazine were administered s.c. after 2·5 mg/kg reserpine i.p. at the time intervals indicated above; death was 3 hr after chlorpromazine. Reserpine alone did not markedly change the HVA content after 4–50 hr.
Each figure indicates an average of 3 experiments ± s.e.

* Chlorpromazine-induced increase of HVA as per cent of that in controls not pretreated with reserpine (absolute increase of HVA of controls: 0·33 ± 0·05 μg/g).
† In percent of untreated controls (absolute values of controls: 1·78 ± 0·05 μg/g).

The exact role of the dopamine stores in the above-mentioned feedback mechanism is not known. One may speculate that dopamine synthesis is regulated by the amount of the amine liberated from the stores. In consequence of blockade of dopaminergic receptors by chlorpromazine, a compensatory discharge of the amine might occur leading to an activation of its synthesis. Such an enhanced liberation of dopamine seems no longer possible if the stores have been emptied by reserpine. Consequently, the feedback mechanism may be impaired. An analogous regulatory mechanism possibly exists for the noradrenergic system, since neuroleptics are also known to block noradrenergic receptors.

In conclusion, experiments with various psychotropic drugs indicate that a relationship between disturbed extrapyramidal function and increased cerebral homovanillic acid levels seems to exist. The storage sites of dopamine may be involved in a feedback mechanism leading to an enhanced formation of homovanillic acid after blockage of dopaminergic receptors by neuroleptics

Medical Research Department,
F. Hoffmann-La Roche & Co. Ltd.,
Basle, Switzerland.
July 15, 1966

M. DA PRADA
A. PLETSCHER

References

Andén, N.-E., Roos, B.-E. & Werdinius, B. (1963). *Life Sci.*, **2**, 448–458.
Andén, N.-E., Roos, B.-E. & Werdinius, B. (1964). *Ibid.*, **3**, 149–158.
Andén, N.-E., Carlsson, A., Dahlström, A., Fuxe, K., Hillarp, N.-Å. & Larsson, K. (1964). *Ibid.*, **3**, 523–530.
Burkard, W. P., Gey, K. F. & Pletscher, A. (1966). *Nature Lond.* In the press.
Carlsson, A. (1964). *Acta neuroveg.* **26**, 484–493.
Carlsson, A. & Lindqvist, M. (1963). *Acta pharmac. tox.*, **20**, 140–144.
Carlsson, A. & Waldeck, B. (1958). *Acta physiol. scand.*, **44**, 293–298.
Gey, K. F. & Pletscher, A. (1964). *J. Pharmac. exp. Ther.*, **145**, 337–343.
Glowinski, J., Iversen, L. L. & Axelrod, J. (1966). *Ibid.*, **151**, 385–399.
Hornykiewicz, O. (1964). *Wien. klin. Wschr.*, **76**, 834.
Juorio, A. V., Sharman, D. F. & Trajkov, T. (1966). *Br. J. Pharmac. Chemother.*, **26**, 385–392.
Laverty, R. & Sharman, D. F. (1965). *Ibid.*, **24**, 759–772.
Pletscher, A., Burkard, W. P. & Gey, K. F. (1964). *Biochem. Pharmac.*, **13**, 385–390.
Da Prada, M. & Pletscher, A. (1966). *Experientia*, **22**, 465–466.
Roos, B.-E. (1965). *J. Pharm. Pharmac.*, **17**, 820–821.
Sourkes, T. L. (1961). *Rev. canad. Biol.*, **20**, 187–196.

The importance of the nervous impulse flow for the depletion of the monoamines from central neurones by some drugs

SIR,—It is known from previous work that the neuronal impulse flow is of great importance for the catecholamine depleting effect of α-methyl-*p*-tyrosine methylester (H 44/68), a potent and selective inhibitor of the enzyme tyrosine hydroxylase, since this drug causes a much more pronounced depletion of noradrenaline from the spinal cord cranial than caudal to a transection (Andén, Corrodi, Dahlström, Fuxe & Hökfelt, 1966). That study was based on the fact that all the 5-hydroxytryptamine (5-HT) and noradrenaline nerve terminals of the spinal cord belong to axons which originate from 5-HT and noradrenaline nerve cell bodies of the lower brain stem (Carlsson, Falck, Fuxe & Hillarp, 1964; Dahlström & Fuxe, 1965). Thus, after total transection of the spinal cord the nerve impulses will reach the monoamine nerve terminals lying cranial but not caudal to the lesion.

This neuronal model has now been utilised to test other drugs inhibiting the 5-HT as well as the catecholamine synthesis. These drugs are α-propyldopacetamide (H 22/54), a potent inhibitor of the 5-HT and catecholamine synthesis (Carlsson, Corrodi & Waldeck, 1963) and H 44/48, a selective inhibitor of the enzyme tryptophan hydroxylase. Furthermore, the effect of reserpine has also been studied, since this drug does not deplete the monoamine stores by synthesis inhibition but by blocking the uptake-storage mechanism of the amine granules (Carlsson, Hillarp & Waldeck, 1963; Dahlström, Fuxe & Hillarp, 1965).

Male, adult rats of 150–250 g have been used. The animals were acutely spinalised in the mid-thoracic region under ether anaesthesia. When the rats had woken up they were injected by the intraperitoneal route with the following drugs: H 22/54 (500 mg/kg, 4 hr before killing), H 44/48 (two doses of 500 mg/kg each, 3 and $1\frac{1}{2}$ hr before killing) and reserpine (1 or 2·5 mg/kg, 4 hr before killing). The animals were kept in a temperature of +29° throughout the experiment to prevent the hypothermic action of the injected drugs.

For histochemical analysis the rats were killed by decapitation under light chloroform anaesthesia. Pieces of the spinal cord cranial and caudal to the site of transection were dissected out, freeze-dried, treated with formaldehyde gas, embedded in paraffin and mounted as previously described in detail (Dahlström & Fuxe, 1964; Hamberger, Malmfors & Sachs, 1965).

For biochemical analysis the rats were killed by a blow on the head. The spinal cord cranial and caudal to the lesion was taken out as quickly as possible. The pia mater and the roots were removed. For the 5-HT experiments the spinal cords from 2 or 3 rats were pooled. The noradrenaline and 5-HT were assayed spectrofluorimetrically after cation exchange chromatography (Häggendal, 1963; Andén & Magnusson, unpublished descriptions).

H 22/54. After injection of the drug H 22/54 to intact rats there was a marked and general decrease of 5-HT and noradrenaline in the 5-HT and noradrenaline nerve terminals of the whole spinal cord as revealed by both histochemical and biochemical determinations of these amines. When the drug was given after total transection, however, the same marked decrease of 5-HT and noradrenaline was observed in the respective nerve terminals cranial to the place of transection, whereas caudal to it both kinds of nerve terminals appeared to have practically normal amine levels. These findings were corroborated by biochemical experiments (Table 1). If an additional dose of H 22/54 was given 4 hr after the first dose the difference between the cranial and caudal half seemed to be even more pronounced.

H 44/48. After injection of H 44/48 to spinalized rats the intensity of the 5-HT nerve terminals as well as the biochemically determined 5-HT (Table 1) was reduced more in the cranial than in the caudal part, whereas in intact rats the decrease was about the same all over the spinal cord. The noradrenaline nerve terminals and the biochemically assayed noradrenaline were not changed.

Reserpine. After administration of reserpine to spinalized rats, with the doses used, there was a more pronounced depletion of the noradrenaline nerve terminals cranial than caudal to the lesion as revealed both histochemically and biochemically (Table 1). However, no significant difference was observed in the reductions of the amine levels of the 5-HT nerve terminals in the cranial and caudal part of the transected spinal cord (Table 1). In intact rats, reserpine produced a similar noradrenaline depletion in all parts of the spinal cord.

The present findings with H 44/48 and especially with H 22/54 show that the neuronal impulse flow is of great importance also for the depletion of 5-HT after inhibition of its synthesis. Thus, it is also possible to study the state of activity

in the 5-HT neurones with the help of potent inhibitors of the 5-HT synthesis in the same way as has been demonstrated for the catecholamine neurones.

TABLE 1. EFFECT OF CERTAIN DRUGS ON THE LEVELS (μg/g; MEAN $\pm$ S.E.) OF NORADRENALINE OR 5-HYDROXYTRYPTAMINE (5-HT) IN THE RAT SPINAL CORD CRANIAL AND CAUDAL TO A TOTAL TRANSECTION. The differences necessary for significance were calculated by analysis of variance. Number of experiments in parentheses.

Drug: dose and time	Amine	Part of the spinal cord	No drug treatment	Drug treatment	Reduction by drug %	Difference
H 22/54	5-HT	Cranial	0·39 $\pm$ 0·015(5)	0·18 $\pm$ 0·031(5)	54	50%
		Caudal	0·68 $\pm$ 0·065(5)	0·65 $\pm$ 0·061(5)	4	($P < 0·001$)
500 mg/kg, hr	Noradrenaline	Cranial	0·27 $\pm$ 0·029(3)	0·12 $\pm$ 0·029(3)	55	38%
		Caudal	0·48 $\pm$ 0·032(3)	0·40 $\pm$ 0·017(3)	17	($P < 0·025$)
H 44/48 500 mg/kg × 2, 3 + 1½ hr	5-HT	Cranial	0·33 $\pm$ 0·014(7)	0·17 $\pm$ 0·016(7)	47	23%
		Caudal	0·74 $\pm$ 0·027(7)	0·57 $\pm$ 0·043(7)	24	($P < 0·01$)
Reserpine	Noradrenaline	Cranial	0·29 $\pm$ 0·015(6)	0·06 $\pm$ 0·007(6)	79	22%
		Caudal	0·42 $\pm$ 0·030(6)	0·18 $\pm$ 0·025(6)	57	($P < 0·01$)
2·5 mg/kg, 4 hr	5-HT	Cranial	0·24 $\pm$ 0·015(5)	0·10 $\pm$ 0·010(5)	59	−6%
		Caudal	0·56 $\pm$ 0·034(5)	0·20 $\pm$ 0·013(5)	65	

The results also show that the amine-depleting effect of reserpine is not dependent on the nervous impulse flow to the same high degree as that of synthesis inhibitors. Thus, the model used may also be of value to find out if an amine depleting drug acts by inhibiting the synthesis or by blocking the granule uptake.

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Department of Pharmacology, University of Göteborg. — N.-E. ANDÉN

Department of Histology, Karolinska Institutet, Stockholm, Sweden. — F. FUXE, T. HÖKFELT

July 21, 1966

References

Andén, N.-E., Corrodi, H., Dahlström, A., Fuxe, K & Hökfelt, T. (1966). *Life Sci.*, 5, 561–568.
Carlsson, A., Corrodi, H. & Waldeck, B. (1963). *Helv. chim. Acta*, 46, 2270–2285.
Carlsson, A., Hillarp, N.-Å. & Waldeck, B. (1963). *Acta physiol. scand.*, **59**, *Suppl.* 215, 1–38.
Carlsson, A., Falck, B., Fuxe, K. & Hillarp, N.-Å. (1964). *Ibid.*, **60**, 112–119.
Dahlström, A. & Fuxe, K. (1964). *Ibid.*, **62**, *Suppl.* 232, 1–55.
Dahlström, A. & Fuxe, K. (1965). *Ibid.*, 64, *Suppl.* 247, 1–36.
Dahlström, A., Fuxe, K. & Hillarp, N.-Å. (1965). *Acta pharmac. tox.*, **22**, 277–292.
Hamberger, B., Malmfors, T. & Sachs, Ch. (1965). *J. Histochem. Cytochem.*, **13**, 147.
Häggendal, J. (1963). *Acta physiol. scand.*, **59**, 242–254.

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