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Research Papers

The flow properties of magnesia

N. PILPEL

A study has been made of the effect of particle size and particle size distribution on the angle of repose of granulated magnesium oxide. Particles smaller than about 100 μ diameter produce a substantial increase in the angle of repose: this has been explained in terms of frictional and van der Waal's cohesive forces.

MEASUREMENT of the angle of repose of a powder yields information on how it flows in comparison with other powders. Angles of 30° or below usually indicate that the flow is "free," angles of 40° or above that it is broken and that the phenomenon of "balling" may occur (Neumann, 1953). Brown (1960), Train (1958), Craik (1958), Miller (1958) and Dallavalle (1948) have shown that the value of the angle of repose that is obtained depends not only on the way in which the cone of powder is produced, but also on the nature of the powder, how it has been prepared, on the size of the particles and on their size distribution. But relatively little work of a quantitative nature has been done to correlate the measured angles of repose with these variables.

A simple new technique has now been developed for forming prepared mixtures of granulated magnesium oxide into cones. A study has been made of the effect of particle size and particle size distribution on the angle of repose and equations have been developed from first principles to explain the observed results in terms of frictional and cohesive forces that act between neighbouring particles.

Experimental

PREPARATION OF SIEVE FRACTIONS

Granulated magnesium oxide, obtained from the Washington Chemical Company, was dried at 800° for 2 hr in the oven. It was separated into narrow fractions by sieving on an Alpine Airjet sieve (Lavino 1964) using 50 g portions and sieving for a standard period of 2 min. The various fractions were stored before use in dry screw-capped bottles and mixtures of the different sized powders were made up by weight.

ANGLE OF REPOSE

(*a*) A few granules from each sieve fraction were placed in turn on a clean, dry glass slide, one end of which was then slowly raised until the granules started to slide. At this point, the elevation of the slide to the horizontal was measured.

The microscopic appearance of the different granules at a magnification of $\times 100$ was noted to see whether there was any significant change in their shape with size.

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The densities of the different sized granules were also determined by the specific gravity bottle method, using toluene and a light mineral oil as the fluids.

(*b*) Preliminary measurements of the angle of repose, in which a fixed volume of powder was allowed to flow through a funnel to form a conical heap, gave variable results. This was due to the occurrence of non-uniform "broken" flow which, in several instances, produced damage to the apex of the cone.

A static method of measurement was therefore devised. A fixed volume of powder was poured into an open brass tube, 1·5 inch in diameter and 2 inches high, standing on a 2 inch diameter brass base. On slowly raising the tube the powder flowed out to form a conical heap on the base. The height, h, of the cone was measured and the angle of repose θ, calculated from the expression

$$\theta = \tan^{-1} \frac{h}{r}$$

where r is the radius of the base.

Since values of θ varying by up to 2° could be produced by using tubes and bases of different sizes, it was necessary to use one apparatus for all the measurements. These were made in sextuplicate, individual determinations were found to be reproducible to $\pm$ 1°, the error in the mean of the six determinations being, therefore, $\pm$0·2°.

Results

The graph relating the size of the particles to the elevation of the slide when sliding commences is given in Fig. 1.

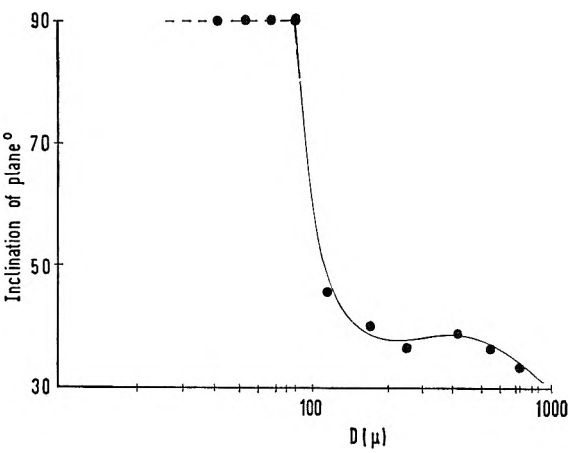

FIG. 1. Inclination of plane (degrees) when sliding of different sized particles commences.

Fig. 2 shows the variation of angle of repose with particle size for narrow sieve cuts.

Fig. 3 shows the effect on the angle of repose of adding various amounts of smaller particles ("fines") to a particular sieve cut, 400 μ in diameter.

Figs 4 (a) and (b) give the corresponding results for the ternary systems.

Examination of Fig. 1 shows that small particles slide less easily down an inclined plane than large ones, sliding ceasing altogether when the particles are less than 90 μ in diameter. There is a slight upward trend in the curve between 350 and 450 μ which may be due to the particular influence of very fine material trapped on the surfaces of particles of this size.

Below the critical size of 90 μ it appears that the weight of a particle of MgO is being completely supported by the force of adhesion which acts between it and the surface of the glass. The effect of particle shape can be discounted since under the microscope the shapes of all the particles in the size range 53 to 725 μ were essentially similar.

Now the density of granular magnesium oxide 90 μ in diameter is about 3 g/cm^3. Its real, as opposed to its apparent area of contact with the glass is probably between 10^{-8} and 10^{-10} times its total surface area (Bowden & Tabor, 1954) implying contact along a line between 10^{-6} and 10^{-7} cm long. Calling γ ergs cm^{-2} the interfacial energy and assuming that the coefficient of friction between the granule and the glass is unity, γ is found to lie between 10^2 and 10^3 erg cm^{-2}. This is in reasonable agreement with the figures given by Gregg (1961) for the surface energies of glass and of magnesia.

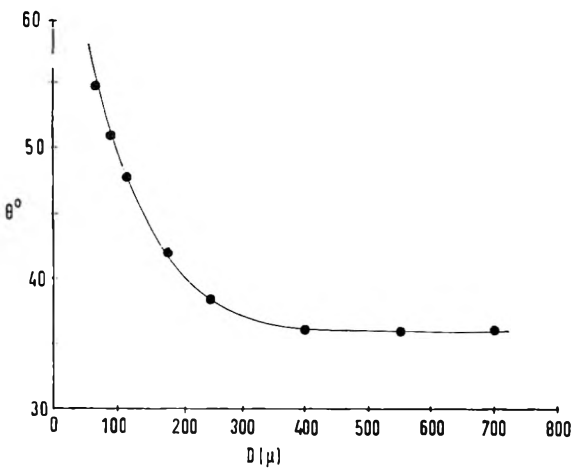

FIG. 2. Angles of repose for different sieve cuts.

Fig. 2 shows that for a powder comprising a narrow sieve cut, the angle of repose is inversely proportional to the size of the powder particles, the relationship being of the form

$$\theta = AD^{-1} + B$$

where D is the particle mean diameter in μ and A and B are constants whose values depend on the nature of the powder and on the suface shape, roughness and so on of the particles. With the present material A = 18×10^3 and B = 32·2.

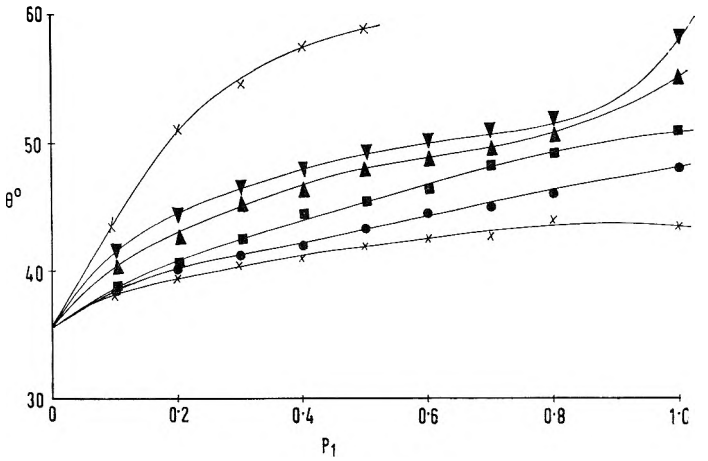

FIG. 3. Effect of fines on powder 400 μ diameter.
$\times$ = 180μ; ● = 125μ; ■ = 90μ; ▲ = 68μ; ▼ = 53μ; upper curve $\times$ = 30μ (average).

Fig. 3 shows that for mixtures of two sieve cuts of which one—designated fines has particles <150 μ in diameter, the angle of repose is inversely

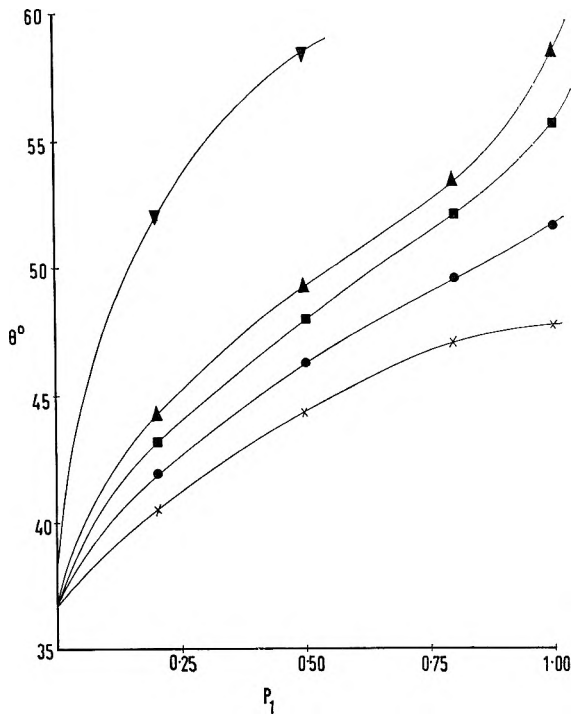

FIG. 4 (a). Effect of fines on mixtures of powder. 20 parts 250μ diameter, 80 parts 725μ diameter.
$\times$ = 125μ; ● = 90μ; ■ = 68μ; ▲ = 53μ; ▼ = 30μ (average).

proportional to the size of the fine particles, but directly proportional to the weight fraction of them present.

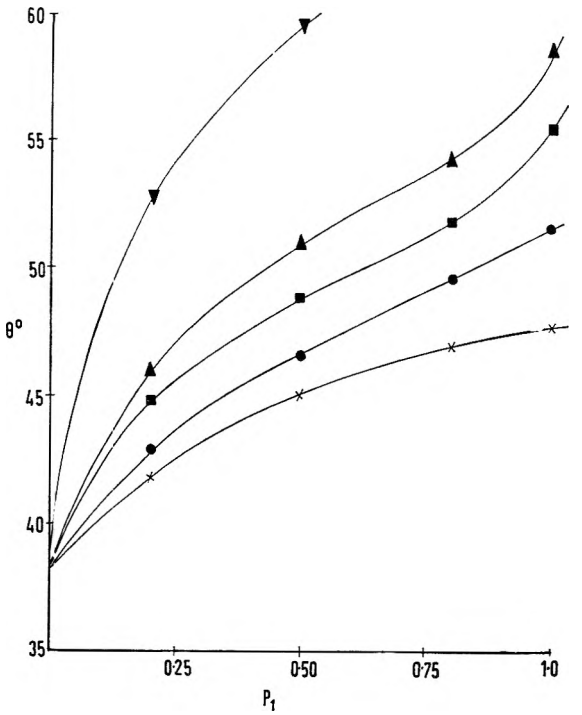

FIG. 4(b). Effect of fines on mixtures of powder. 50 parts 250μ diameter 50 parts 725 μ diameter. Key as Fig. 4 (a).

Figs 4 (a) and (b) show that for mixtures of three sizes of powder in which at least one has particles less than 150 μ in diameter the angle of repose is a function of the ratio of the linear dimensions D_1, D_2, D_3 of each size of particle, and also a function of their weight fractions P_1, P_2, P_3.

$$\text{i.e. } \theta = f(D_1 : D_2 : D_3, P_1 : P_2 : P_3)$$

Discussion

It is well known that the presence of fines can have a considerable effect on the angle of repose of a powder and in the present systems as little as 0·05 weight fraction increases θ by between 2 and 5°. This can be presumed to be due to the enhancement of the forces that normally operate between the particles concerned.

Five types of force may be postulated as acting between particles in a powder. Firstly, the force of friction. Secondly, surface tension forces due to the possible presence on the particles of adsorbed films of gas and/or moisture (Gregg, 1961) in spite of the precautions taken in their preparation. Thirdly, mechanical forces caused by interlocking of particles of irregular shape. Fourthly, electrostatic forces which arise from friction

between particles (Green & Lane, 1957). Fifthly, cohesive or van der Waal's forces which operate between neighbouring molecules (Green & Lane, 1957).

But of these the surface tension and mechanical forces are probably small since it is known that spherical particles often exhibit greater cohesion than irregular particles both before and after degassing. The electrostatic forces are forces of repulsion which, even for particles in the size range 1 to 10 μ do not exceed about $D_t \times 10^{-8}$ dynes per particle (Kunkel, 1950) and which, for larger particles of MgO with a density circa 3·2 g/cm^3 become negligible in comparison with their weight.

There remain friction and the van der Waal's forces of cohesion.

Bradley (1936), Hamaker (1937) and Jordan (1954) have considered the magnitude of the van der Waal's forces for powders of different materials, making allowance for the fact that the particles may be non-spherical in shape and have appreciable surface irregularities. For magnesium oxide they appear to be of the order $D \times 10^{-6}$ dynes per particle, i.e. large in comparison with any electrostatic forces and when the particles are about 30 μ in diameter the cohesive forces become comparable to their weight.

Let it therefore be assumed that it is friction and the van der Waal's forces which predominate and which are primarily responsible for the effects produced by fines on the angle of repose of magnesia powder.

In the simplest instance of a particle of MgO of mass m starting to slide down the inclined surface of a cone of identical particles

$$mg \sin \theta = \mu (mg \cos \theta + F)$$

or
$$F/mg = \left(\frac{\sin \theta}{\mu} - \cos \theta\right) \quad .. \quad .. \quad .. \quad (1)$$

Where θ is the elevation of the cone (angle of repose).

μ is the coefficient of friction

and F is the cohesive force between the particle and its neighbours.

For large particles F becomes negligible in comparison with mg and

$$\mu = \tan \theta_{11m} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

Where θ_{11m} is the angle of repose of coarse particles.
Substituting into equation (1)

$$F/mg = \left(\frac{\sin \theta}{\tan \theta_{11m}} - \cos \theta\right) \quad .. \quad .. \quad .. \quad (3)$$

Now consider the cone to be composed of particles of M different sizes, their linear dimensions $D_1, D_2, D_3 \ldots D_r \ldots D_M$ being such that

$$D_1 << D_2 << \ldots D_r << \ldots D_M$$

For convenience the particles are assumed to be small cubes. An r-sized particle (designated an r-particle) is pictured as occupying a cell (an r-cell) having the shape of a rectangular parallelopiped whose dimensions $a_r \times a_r \times X_r$ are relatively large in comparison with D_r. Each r-particle is surrounded by a number n_r (r–1) cells measuring $a_{r-1} \times a_{r-1} \times X_{r-1}$, each of which in turn contains an (r–1) particle and n_{r-2} (r–2)-cells. The

smallest cell is a 2-cell, the smallest particle is a 1-particle and $D_1 << D_2, a_2 << \ldots D_r, a_r << \ldots D_M, a_M$.

It is possible to derive expressions relating the angle of the cone to the friction and the cohesive forces exerted by the 1-particles in it.

Let P_r be the weight fraction of r-particles

δ_r the apparent density of powder in an r-cell

m_r the mass of powder in an r-cell

n_r the number of r-cells in an (r + 1)-cell

D_r the linear dimension of an r-particle, so that its volume is D_r^3

δ the density of any particle.

The mass of powder in an r-cell is given by

$$m_r = a_r^2 \times X_r \delta_r = (D_r^3 + n_{r-1} m_{r-1}) \quad .. \quad .. \quad (4)$$

and

$$\frac{P_r}{\sum_{i=1}^{r-1} P_i} = \frac{D_r^3 \delta}{n_{r-1} m_{r-1}} \quad .. \quad .. \quad .. \quad .. \quad (5)$$

from equations (4) and (5) it follows that

$$m_r = \frac{D_r^3 \delta}{P_r} \sum_{i=1}^{r} P_i \quad .. \quad .. \quad .. \quad .. \quad (6)$$

Now in an r-cell of volume $a_r^3 X_r$ there is one r particle with a volume of D_r^3 and n_{r-1} (r–1)-cells. So the average volume of an (r–1)-cell is $\frac{1}{n_{r-1}} (a_r^2 X_r - D_r^3)$. But only those (r–1)-cells that are situated on the base of the r-cell come into contact with the r-cell below which is supporting it on the inclined plane. The number N_r of (r–1)-cells contributing to cohesion between two adjacent r-cells is thus

$$N_r = \frac{a_r^2}{\left[\frac{1}{n_{r-1}} (a_r^2 X_r - D_r^3)\right]^{\frac{2}{3}}} \quad .. \quad .. \quad .. \quad (7)$$

Employing equations (4) and (6) this can be rewritten

$$N_r = \frac{a_r^2}{\left[\frac{1}{n_{r-1}} D_r^3 \left(\frac{\delta}{\delta_r} \frac{\sum_{i=1}^{r} P_i}{P_r} - 1\right)\right]^{\frac{2}{3}}}$$

$$= \frac{D_r^3 \delta}{P_r X_r \delta_r \left[\frac{1}{n_{r-1}} D_r^3 \left(\frac{\delta}{\delta_r} \frac{\sum_{i=1}^{r} P_i}{P_r} - 1\right)\right]^{\frac{2}{3}}}$$

$$= \frac{\delta D_r \sum_{i=1}^{r} P_i}{X_r \delta_r P_r} \left[\frac{1}{n_{r-1}} \left(\frac{\delta}{\delta_r} \frac{\sum_{i=1}^{r} P_i}{P_r} - 1\right)\right]^{-\frac{2}{3}}$$

But from equation (5) and using equation (6)

$$\frac{1}{n_{r-1}} = \frac{P_r}{\sum_{i=1}^{r-1} P_i} \frac{m_{r-1}}{D_r^3 \delta} = \frac{P_r D_{r-1}^3}{P_{r-1} D_r^2}$$

Thus

$$N_r = \frac{\delta D_r^3 \sum_{i=1}^{r} P_1}{\delta_r P_r X_r D_{r-1}^2} \left[\frac{1}{P_{r-1}} \left(\frac{\delta}{\delta_r} \sum_{i=1}^{r} P_1 - P_r \right) \right]^{-\frac{2}{3}} \quad .. \quad (8)$$

The number, N, of smallest particles, i.e. 1-particles contributing to the cohesive force between an M-cell face and the inclined plane is

$$N = N_2 N_3 N_4 \ldots N_M \quad .. \quad .. \quad .. \quad (9)$$

Hence

$$N = \delta \prod_{r=2}^{M} \frac{D_r^3 \sum_{i=1}^{r} P_1}{\delta_r P_r X_r D_{r-1}^2} \left[\frac{1}{P_r} \left(\frac{\delta}{\delta_r} \sum_{i=1}^{r} P_1 - P_r \right) \right]^{-\frac{2}{3}} \quad (10)$$

These particles produce a total cohesive force F which is known to be proportional to their linear dimension and which can be assumed to be proportional to the number of them, n, present in each 2-cell. Writing this in the form

$$\frac{F}{mg} = \frac{kND_1^n}{m_M g} \quad .. \quad .. \quad .. \quad .. \quad (11)$$

where k is a constant and employing equations (6) and (10) it follows that

$$\frac{F}{mg} = \frac{k}{g} \frac{P_M D_1^n}{D_M^3} \prod_{r=2}^{M} \frac{D_r^3 \sum_{i=1}^{r} P_1}{\delta_r P_r X_r D_{r-1}^2} \left[\frac{1}{P_{r-1}} \left(\frac{\delta}{\delta_r} \sum_{i=1}^{r} P_i - P_r \right) \right]^{-\frac{2}{3}} \quad (12)$$

This is the general equation relating friction and the cohesive force to the numbers and sizes of the different particles in the powder. Substituting from equation (3) in order to introduce the measured angle of repose it follows that

$$\left(\frac{\sin \theta}{\tan \theta_{11m}} - \cos \theta \right) = \phi = \frac{k}{g} \frac{P_M D_1^n}{D_M^5} \prod_{r=2}^{M} \frac{D_r^3 \sum_{i=1}^{r} P_1}{\delta_r P_r X_r D_{r-1}^2} \left[\frac{1}{P_{r-1}} \left(\frac{\delta}{\delta_r} \sum_{i=1}^{r} P_i - P_r \right) \right]^{-\frac{2}{3}} \quad .. \quad (13)$$

We can now consider several special cases.

(*a*) *Powder containing only one size of particle*

Here $M = 1$ $P_1 = 1$. Although it does not follow formally from equation (3) it is apparent that

$$\phi = \frac{k}{g} D_1^{n-3} \quad .. \quad .. \quad .. \quad .. \quad (14)$$

which may be compared to the empirical expression for θ already obtained.

(*b*) *Powder containing "fines" and one other particle size*

Here $M = 2$, $P_1 + P_2 = 1$ and equation (13) reduces to

$$\phi = \frac{k}{g}\frac{D_1^{n-2}}{\delta_2 X_2}\left[\frac{P_1}{(\delta/\delta_2 - P_2)}\right]^{\frac{2}{3}} \quad .. \quad .. \quad .. \quad (15)$$

(*c*) *Powder containing "fines" and two other particle sizes* $D_1 << D_2 << D_3$

Here $M = 3$, $P_1 + P_2 + P_3 = 1$ and equation (13) becomes

$$\phi = \frac{k}{g}\frac{P_3 D_1^n}{D_3^3}\left\{\frac{D_2^3(1 - P_3)}{\delta_2 P_2 X_2 D_1^2}\left[\frac{P_1}{\delta/\delta_2(1 - P_3) - P_2}\right]^{\frac{2}{3}}\right. \cdot \left\{\frac{D_3^3}{\delta_3 P_3 X_3 D_2^2}\left[\frac{P_2}{\delta/\delta_3 - P_3}\right]^{\frac{2}{3}}\right.$$

$$= \frac{k}{g}\frac{D_1^{n-2} D_2 (1 - P_3)(P_1 P_2)^{\frac{2}{3}}}{\delta_2 \delta_3 X_2 X_3 P_2\left[\delta/\delta_2(1 - P_3) - P_2\right]^{\frac{2}{3}}\left[\frac{\delta}{\delta_3} - P_3\right]^{\frac{2}{3}}} \quad .. \quad (16)$$

(*d*) *Powder containing "fines" and two other particle sizes* $D_1 << D_2 < D_3$

Here a weighted mean size is taken for D_2 and D_3 and the problem then reduces to case (*b*) above.

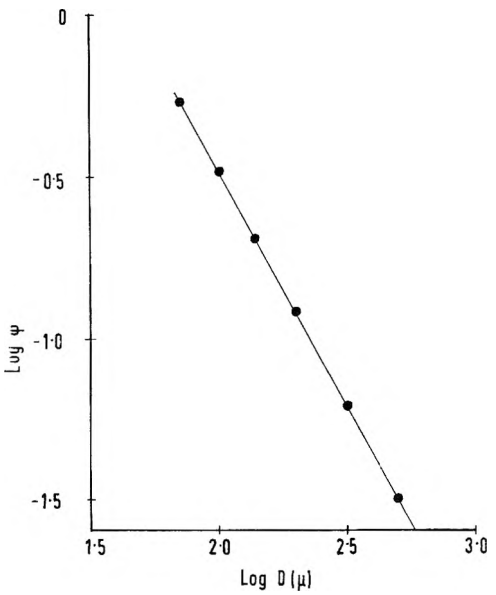

FIG. 5. Single component system. Log ϕ versus log D.

It is seen that in case (*a*) ϕ should be proportional to D_1^{n-3}, in all the other cases to D_1^{n-2}. (Considerations of symmetry show that ϕ should remain proportional to D_1^{n-2} for higher values of M also, although an

increasing correction will now be required to allow for density differences of the powder in the different sized cells.) It follows that straight line graphs should be obtained when log ϕ is plotted against log D_1, the slopes of the lines yielding values for n. This prediction has been tested in Figs. 5–8 for the data on the single component system (contained in Fig. 2) for the data on the binary system (contained in Fig. 3) and for the data on the ternary systems (contained in Figs 4 (a) and (b).) It is seen that in all instances the agreement with prediction is very good.

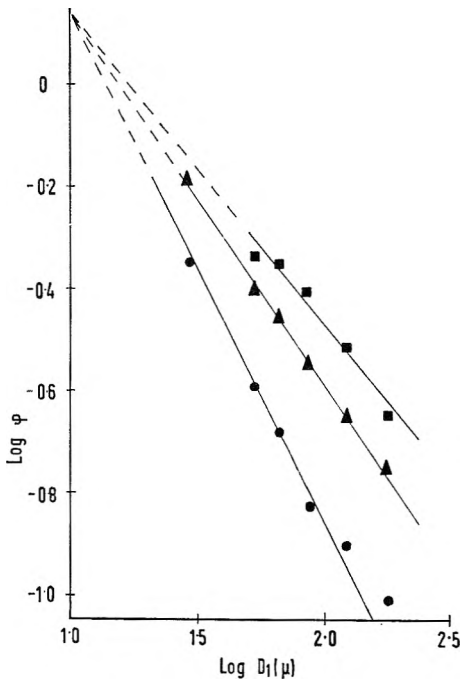

FIG. 6. Binary systems. Log ϕ versus log D_1.
■ $P_1 = 0{\cdot}8$; n = 1·39 ▲ $P_1 = 0{\cdot}5$; n = 1·28 ● $P_1 = 0{\cdot}2$; n = 1·00.

As the weight fraction of fines in a binary or a ternary system is increased, so the value of n increases, the relationship being of the form

$$n = c \log P_1$$

where c is a constant and P_1 as before is the weight fraction of fines. This results in divergence of the plots of log ϕ versus log D_1 from a virtual common origin for each system.

The ordinate of the origin yields a value for ϕ and since θ_{11m} for the system is known, θ at this point can be calculated. The values turn out to be exactly 90° both for the binary systems and for the ternary systems.

Theoretically, therefore, the origins may be pictured as representing idealised combinations of particle sizes when the angle of repose should become 90° and the powder ceases to flow (for the binary systems this would be when the fines were 10·5 μ in diameter, for the ternary systems

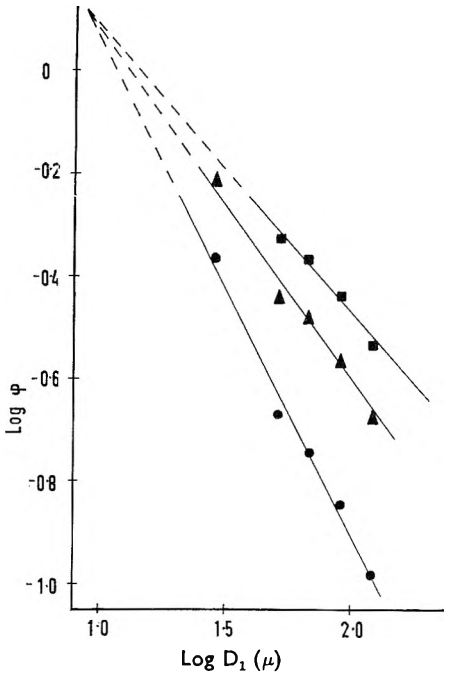

FIG. 7. Ternary systems. (20/80 mixture). Log ϕ versus log D_1.
■ $P_1 = 0{\cdot}8$; $n = 1{\cdot}44$ ▲ $P_1 = 0{\cdot}5$; $n = 1{\cdot}32$ ● $P_1 = 0{\cdot}2$; $n = 1{\cdot}04$.

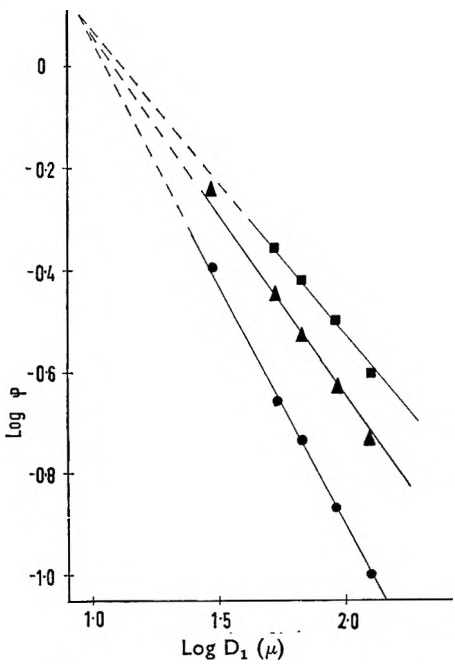

FIG. 8. Ternary systems. (50/50 mixture). Log ϕ versus log D_1.
■ $P_1 = 0{\cdot}8$; $n = 1{\cdot}41$ ▲ $P_1 = 0{\cdot}5$; $n = 1{\cdot}28$ ● $P_1 = 0{\cdot}2$; $n = 1{\cdot}05$.

when they were 8·9 μ in diameter, the total mass of powder in each system being small in comparison with the cohesive forces).

For real systems, however, where the mass of powder must be large in comparison with the cohesive forces if the angle of repose is to be measured, this idealized situation does not occur. The powder continues to flow, albeit in a broken column, and the plots of log ϕ versus log D_1 in Figs 6–8 must therefore depart from linearity when the values of D_1 are made very small.

It is hoped to be able to test this prediction in due course.

Going back to the general equation (13) relating friction and the cohesive force to the number and sizes of the different particles in the powder, it is seen that the intercept on the ordinate of the graph of log ϕ versus log D_1 should yield a value for the sum of the logarithms of all the other terms in the equation. If the values of P_r, δ_r and X_r, etc. were known, this would enable k, the force constant, to be calculated.

However, it is more convenient to determine k from equation (14) which contains no other unknown quantities. From the intercept on the ordinate of Fig. 5, k is found to have the numerical value of 2·14 when D is measured in cm and g is 981 cm sec^{-2}.

Acknowledgements. The author wishes to thank Mr. M. Solomons for making some of the measurements and Mr. P. Heyda for mathematical assistance.

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Genotropic agents : steroid basic ethers

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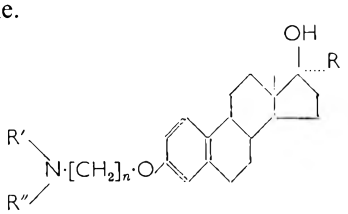
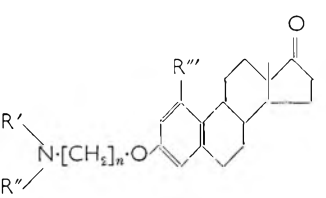
The synthesis and biological activity of a series of basic ethers of the steroid oestrogens is described. The compounds have genotropic (Black, 1961) activity coupled with low oestrogenicity.

THE problem of whether the pituitary-suppressant activity of steroidal oestrogens is due to their oestrogenicity or the result of their molecular structure has not been satisfactorily answered (Dodds, 1961). No aromatic ring-A steroid devoid of oestrogenicity has, so far, shown pituitary-suppressant activity. We have now synthesised a series of basic ethers (I, II see Tables) having low oestrogenicity coupled, in some cases, with anti-gonadotrophic activity in rats equal to that of norethisterone.



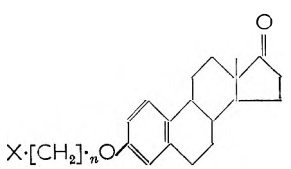
(I)



(II)

The ethers (I) (Table 1) were prepared by reaction of a suitable 17α-substituted oestradiol with the appropriate ω-dialkylaminoalkyl chloride hydrochloride in the presence of alkali, and a similar reaction using oestrone in place of the oestradiol derivatives gave compounds (II) (Table 2). An alternative method used for the synthesis of the oestrone derivatives (II) was the reaction of the appropriate amine with a ω-haloalkyl ether (III), which was obtained by reaction of oestrone with a αω-dihaloalkane. The oestradiol analogues (I; R = H) were prepared by reduction of the corresponding 17-keto-derivative with potassium borohydride in methanol.

The ethers (I, R = R″ = H; R′ = Me; $n = 2$) and (I, R = R′ = R″ = H; $n = 4$) were also prepared; the former by debenzylation of I (R = H; R′ = $C_6H_5 \cdot CH_2$; R″ = Me; $n = 2$) and the latter by reduction of 3-(ω-cyanopropoxy)oestra-1,3,5(10)-trien-17-one (IV) with lithium aluminium hydride.



III, X = I, Br or Cl
IV, X = CN; $n = 3$

From the Research Department, Parke Davis & Co., Hounslow, Middlesex.

BIOLOGICAL ACTIVITIES

The biological activities of these compounds have been assessed by our colleagues in Ann Arbor. Their findings are here summarised.

Initially the compounds described were examined for their ability to completely prevent litters being born to mice under conditions where 50–100% of the mice in a control group produced litters. Oestrogenicity was determined either by a rat vaginal cornification (Allen & Doisy, 1923) or mouse uterine weight assay (Evans, Varney & Koch, 1941). Most of the basic ethers showed an oestrogenicity equivalent to 0·05–0·5% that of stilboestrol.

The compounds most extensively examined were of type (I) and (II) in which $n = 3$, and in this series the 17-ketone (II, $R' = R'' = Me$; $R''' = H$; $n = 3$), administered orally in 0·7–1·4 mg/kg of body weight, prevented pregnancy in mice. Replacement of one *N*-methyl group by a benzyl or phenyl group produced only marginal differences in genotropic activity, whereas introduction of a methyl group in position 1 in the steroid molecule eliminated genotropic activity. Substitution at position 17 also produced some differences in degree of genotropic activity. Thus, in Table 1, the 17α-ethyl compound (4), 17α-ethynyl compound (9) and 17α-allyl compound (17) were less active than the 17α-methyl (2) and 17α-vinyl (7) compounds.

An increase in length of the alkyl chain, as in the 17-ketone (21, Table 2), produced little change in genotropic activity. Compounds of type (I) and (II) in which $n = 2$ were almost invariably less active than the corresponding compounds with $n = 3$, although the oestrogenicity of both series was similar.

Evidence suggesting an effect on the pituitary is that the compound 19 (Table 2), administered orally, had an activity greater than norethisterone in suppressing gonadotrophin-induced ovulation in rats (Callantine, Humphrey & French, 1962; Callantine, Lee, Humphrey & Windsor, 1964). This anti-gonadotrophic activity is coupled with an oestrogenicity of 0·3% that of stilboestrol. The compound 19 has no progestational or anti-oestrogenic activity.

The haloalkyl ethers (III) also showed genotropic activity coupled, however, with a relatively high oestrogenicity (1·7–7·1% that of stilboestrol).

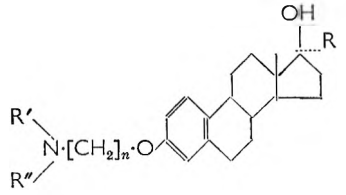
Experimental

Melting-points were determined on a Kofler block. Specific rotations are for chloroform solutions (unless indicated otherwise) at room temperature. All the compounds described exhibited infra-red spectra consistant with the assigned structures.

GENERAL METHODS FOR PREPARATION OF THE BASIC ETHERS

(*a*) *From the phenol.* A mixture of the phenolic steroid (10 mmole), ω-dialkylaminoalkyl chloride hydrochloride (11 mmole), potassium hydroxide (21–22 mmole, as 5N or 10N aqueous solution) in a convenient

TABLE 1. BASIC ETHERS OF 17α-SUBSTITUTED OESTRADIOL.



Comp. No.	R	R′	R″	n	Yield (%)	m.p.°C	Crystallising solvent	$[\alpha]_D^{\circ}$	c	Formula	Found C	Found H	Found Cl	Required C	Required H	Required Cl
1	Me	Me	Me	2	56	100–101·5	n-hexane	+42	1·12	$C_{23}H_{39}NO_2$	77·0	9·65		77·3	9·9	
2	Me	Me	Me	3	38	80–82 & 85–86	n-hexane	+44	0·92	$C_{24}H_{41}NO_2$	77·7	10·0		77·6	10·0	
3	Et	Me	Me	2	21	66–68	n-hexane	+43	0·96	$C_{24}H_{41}NO_2$	77·2	10·0		77·6	10·0	
4	Et	Me	Me	3	30	107–108	ether–n-hexane	+43	1·20	$C_{25}H_{43}NO_2$	78·2	10·2		77·9	10·2	
5	Et	Me	$C_6H_5{\cdot}CH_2$	2	54	oil	—	+28	1·06	$C_{30}H_{41}NO_2$	80·5	9·2		80·5	9·2	
6	$CH_2{:}CH$	Me	Me	2	41	108–110	n-hexane	+47	0·95	$C_{24}H_{39}NO_2$	77·8	9·7		78·0	9·55	
7	$CH_2{:}CH$	Me	Me	3	32	83·5–85·5	n-hexane	+44	1·03	$C_{25}H_{41}NO_2$	78·1	9·7		78·3	9·7	
8	CH:C	Me	Me	2	30‡	162–164·5	methanol	+10	1·09	$C_{24}H_{37}NO_2$	78·3	9·3		78·4	9·05	
9	CH:C	Me	Me	3	49‡	243–247*	ether–ethanol	−8	0·476†	$C_{25}H_{38}ClNO_2$	71·9	9·0	8·5	71·8	8·7	8·5
10	CH:C	Me	Me	3	23‡	154–156	acetone	+6	1·12	$C_{25}H_{39}NO_2$	78·8	9·2		78·7	9·25	
11	CH:C	Me	$C_6H_5{\cdot}CH_2$	2	53	217–219*	isolated direct	+3	0·915†	$C_{30}H_{40}ClNO_2{\cdot}\frac{1}{2}H_2O$	73·3	7·9	7·45	73·6	7·8	7·3
12	CH:C	Me	$C_6H_5{\cdot}CH_2$	3	75‡	89–91	acetone–n-hexane	+3·5	1·04	$C_{31}H_{41}NO_2$	81·7	8·5		81·4	8·6	
13	CH:C	Et	Et	2	70‡	120–121	acetone	+7	0·985	$C_{26}H_{41}NO_2$	78·7	9·8		78·9	9·4	
14	CH:C	Et	Et	3	53	86·5–88·5	n hexane	−0 5	0 655	$C_{27}H_{43}NO_2$	79·35	9·5		79·2	9·6	
15	Pr^n	Me	Me	3	40	208–210*	(sublimed)	+36	1·03†	$C_{28}H_{46}ClNO_2$	71·9	9·6	8·4	71·6	9·7	8·1
16	$CH_2{:}CH{\cdot}CH_2$	Me	Me	2	61	216–219*	(sublimed)	+58	1·025†	$C_{28}H_{44}ClNO_2$	71·5	9·3	8·7	71·5	9·1	8·4
17	$CH_2{:}CH{\cdot}CH_2$	Me	Me	3	33	72–74	n-hexane	+52	0·975	$C_{29}H_{43}NO_2$	78·6	9·45		78·5	9·9	

* hydrochloride salt. † in ethanol.

volume (usually 30–100 ml) of water, ethanol or aqueous ethanol was refluxed for 30–60 min, cooled, diluted with water, and the product extracted with ether. The ethereal extract was diluted with an equal volume of benzene, and the solution washed with Claisen's alkali, washed with water until neutral, and the dialkylaminoalkyl ether extracted with aqueous citric acid solution. After washing with ether the aqueous solution was made alkaline with 2N sodium hydroxide, and the free base extracted with ether. The washed and dried ethereal extract was evaporated to dryness to yield the dialkylaminoalkyl ether. In several instances, indicated ‡ in the tables, the additional purification by extraction with citric acid was omitted.

Compounds of Table 1 and some of those in Table 2 were prepared by this method as were the following 3-ethers of 1-methyl-6-dehydro-oestrone: (*3-dimethylaminopropyl*) *ether* (14%)), double m.p. 111–112° and 123–125° (from methanol), $[\alpha]_D$–84° (*c*, 0·92). Found: C, 78·5; H, 8·8; N, 3·8. $C_{24}H_{33}NO_2$ requires C, 78·4; H, 9·05; N, 3·8%, and [*2*-(N-*benzyl*-N-*methylamino*)*ethyl*] *ether* (70%), m.p. 101–102° (from methanol), $[\alpha]_D$–74° (C, 0·975). Found: C, 81·2; H, 8·1; N, 3·4. $C_{29}H_{35}NO_2$ requires C, 81·1; H, 8·2; N, 3·3%.

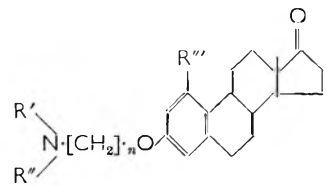
(*b*) *From the ω-haloalkyl ether.* A mixture of a 3-(ω-haloalkoxy)-oestra-1,3,5(10)-trien-17-one and a large excess of the appropriate amine was either refluxed in ethanol for 18 hr or, with the more volatile amines, heated at 50° in benzene in a sealed tube for 24–36 hr before working up as described under (*a*). In certain instances (Compounds 25; 29 and 30; Table 2) where the citrates of the product were not readily soluble in water, purification was achieved by acidifying the ethereal extract with hydrochloric acid, filtering off the hydrochloride, washing it thoroughly with ether and water, and again liberating the free base with alkali and extracting with ether.

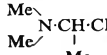
General method for 3-(*ω-haloalkoxy*)*oestra*-1,3,5(10)-*trien*-17-*one.* A solution of oestrone (50 mmole) in ethanol (150 ml) containing 5N potassium hydroxide (50 mmole) was added over 1½–2½ hr, to a refluxing solution of the αω-dihaloalkane (500 mmole) in ethanol (150 ml), and refluxing continued for a further 1–2 hr. The reaction mixture was cooled, poured into water, the product extracted with benzene, and the extract washed with water. With steam-volatile dihaloalkanes (C_3–C_6) the benzene extract was steam distilled, and the residue re-extracted with benzene. The extract was washed with Claisen's alkali, washed with water until neutral, dried, filtered and evaporated to dryness. Crystallisation of the residue from the solvents indicated (Table 3) gave the 3-(ω-haloalkoxy)oestra-1,3,5(10)-trien-17-one.

When 1,10-dibromodecane was used in the above reaction the molar excess was reduced to 225 mmole, the steam distillation omitted, and the product, obtained after washing the benzene extract with Claisen's alkali, was purified by chromatography on Woelm neutral alumina.

3-(3-*Dimethylaminopropoxy*)*oestra*-1,3,5(10)-*trien*-17β-*ol.* A solution of 3-(3-dimethylaminopropoxy)oestra-1,3,5(10)-trien-17-one (2 g) in methanol (50 ml) was stirred for 60 min with potassium borohydride (1 g), the

TABLE 2. BASIC ETHERS OF OESTRONE.



Comp. No.	R′	R″	*n*	R‴	Prep. by method	Yield (%)	m.p. °C	Crystallised from	$[\alpha]_D^{\bullet}$	*c*	Formula	Found C	Found H	Found Cl	Required C	Required H	Required Cl
18	Me	Me	2	H	(a)‡	15	113–114·5	acetone	+132	0·99	$C_{23}H_{31}NO_2$	77·45	9·3		77·4	9·15	
19	Me	Me	3	H	(a)‡	34	90–91°	n-hexane	+127	1·025	$C_{23}H_{33}NO_2$	77·9	9·5		77·7	9·4	
20	Me	Me	4	H	(b)	61	238–240*	ethanol	+119†	0·545	$C_{24}H_{36}ClNO_2$	70·8	9·1	8·6	71·0	8·9	8·7
21	Me	Me	5	H	(b)	54	214–216*	ether–ethanol	+110‡	0·970	$C_{25}H_{38}ClNO_2$	71·5	9·0	8·5	71·3	9·0	8·6
22	Me	Me	6	H	(b)	50	68–69	n-hexane	+112	1·040	$C_{26}H_{39}NO_2$	78·3	9·9		78·5	9·9	
23	Me	Me	10	H	(b)	74	82–84	n-hexane	+83	0·96	$C_{30}H_{47}NO_2$	79·4	10·4		79·4	10·4	
24	Me	$C_6H_5CH_2$	2	H	(a)‡	55	86–88	n-hexane	+105	1·08	$C_{29}H_{35}NO_2$	80·2	8·4		80·5	8·5	
25	Me	$C_6H_5CH_2$	3	H	(a)	54	65–67	n-hexane	+102	1·015	$C_{30}H_{37}NO_2$	80·4	8·5		80·7	8·6	
26	Me	Ph	3	H	(b)	85	99–102	di-isopropyl ether	+104	0·98	$C_{29}H_{35}NO_2$	80·7	8·4		80·5	8·5	
27	Me	Me	3	Me	(a)	41	89–91	n-hexane	+198°	0·97	$C_{24}H_{35}NO_2$	78·0	9·5		78·0	9·55	
28	Me	$C_6H_5·CH_2$	3	Me	(a)	60	94–95·5	n-hexane	+174	0·945	$C_{31}H_{37}NO_2$	80·6	8·7		80·7	8·6	
29	$C_6H_5·CH_2$	H	3	H	(b)	20	215–222*	ethanol	+101†	0·975	$C_{29}H_{36}ClNO_2$	74·5	8·1	7·7	74·1	8·0	7·8
30	$C_6H_5·CH_2$	$C_6H_5·CH_2$	3	H	(b)	72	131–133	acetone	+87	0·935	$C_{35}H_{41}NO_2$	82·6	8·2		82·8	8·1	
31			2	H	(a)‡	58	112–115	ether	+118	0·985	$C_{25}H_{33}NO_2$	78·8	9·35		78·7	9·25	
32			2	H	(a)‡	78	107–109	ethyl acetate	+113	1·05	$C_{24}H_{33}NO_2$	75·1	9·0		75·2	8·7	
33				H	(a)‡	73	70–73	n-hexane	+125	0·97	$C_{28}H_{33}NO_2$	77·4	9·6		77·7	9·4	

* hydrochloride salt † in ethanol

reaction mixture acidified with acetic acid, then made alkaline with 2N sodium hydroxide, and extracted with ether. Evaporation of the washed and dried ethereal extract gave a white solid (2 g), which was crystallised from n-hexane to yield 3-(3-dimethylaminopropoxy)oestra-1,3,5(10)-trien-17β-ol, m.p. 91–93°, $[\alpha]_D$ + 60° (*c*, 0·9 in ethanol). Found: C, 77·4; H, 10·0, N, 3·8. $C_{23}H_{35}NO_2$ requires C, 77·3, H, 9·6, N, 3·9%.

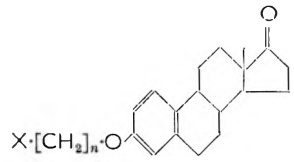
The following compounds were prepared in a similar manner: 3-[2-(N-*Benzyl*-N-*methylamino*)*ethoxy*]*oestra*-1,3,5(10)-*trien*-17β-*ol hydrochloride* (75%), m.p. 194–198° (from ether–ethanol), $[\alpha]_D$ + 50° (*c* 1·045 in ethanol). Found: C, 73·9; H, 8·5; N, 3·1. $C_{28}H_{38}ClNO_2$ requires C, 73·8; H, 8·35; N, 3·1%. 3-(2-*Diethylaminoethoxy*)*oestra*-1,3,5(10)-*trien*-17β-*ol hydrochloride* (76%), m.p. 215–220° (from ether-ethanol), $[\alpha]_D$ + 60°. Found: C, 70·9; H, 9·5; Cl, 8·6; N, 3·2. $C_{24}H_{38}ClNO$ requires C, 70·6; H, 9·4; Cl, 8·7; N, 3·4%. This compound, acetylated with acetic anhydride in pyridine, gave the corresponding 17-*acetate*, m.p. 177–181° (from ether–ethanol), $[\alpha]_D$ + 30° (*c*, 0·75 in ethanol). Found: C, 65·3; H, 9·2; Cl, 7·6. $C_{26}H_{40}ClNO_3$. $2\frac{1}{2}$ H_2O requires C, 65·1; H, 9·3; Cl, 7·4%.

3-(2-*Methylaminoethoxy*)*oestra*-1,3,5(10)-*trien*-17-*one*. 3-[2-(*N*-benzyl-*N*-methylamino)ethoxy]oestra-1,3,5(10)-trien-17-one hydrochloride (1 g) was hydrogenated in ethanol over palladised charcoal (10%, 500 mg), the catalyst filtered off, and the filtrate evaporated to dryness. The residue was made alkaline, extracted with ether, and the ethereal extract washed with water, dried, filtered and concentrated to 20 ml. Ethereal hydrogen chloride was added, and the precipitated hydrochloride filtered off. Crystallisation from ethanol gave 3-(2-methylaminoethoxy)oestra-1,3,5(10)-trien-17-one hydrochloride (90%), m.p. 258–263°. Found: C, 69·4; H, 8·2; Cl, 9·7; N, 3·8. $C_{21}H_{30}ClNO_2$ requires C, 69·3; H, 8·3; Cl, 9·7; N, 3·85%. The hydrochloride was converted to the free base, m.p. 80–82°, $[\alpha]_D$ + 134° (*c*, 0·985). Found: C, 77·3; H, 8·7. $C_{21}H_{29}NO_2$ requires C, 77·0; H, 8·9%.

3-(4-*Aminobutoxy*)*oestra*-1,3,5(10)-*trien*-17β-*ol*. A solution of 3-(3-cyanopropoxy)oestra-1,3,5(10)-trien-17-one (4 g) in ether (200 ml) and tetrahydrofuran (150 ml) was added over 60 min to a stirred solution of lithium aluminium hydride (4 g) in ether (500 ml) under nitrogen. After 60 min under reflux, the reaction mixture was cooled and treated successively with water (6 ml), 15% aqueous sodium hydroxide solution (6 ml) and water (18 ml), the precipitate filtered off, and washed with ether. The filtrate was washed with water, dried with $MgSO_4$, filtered, and evaporated to yield an oil (4 g), which on trituration with ether gave a white solid, m.p. 105–118°. The solid was converted to its hydrochloride, m.p. 210–213° (from ethanol), $[\alpha]_D$ + 61° (*c*, 0·865 in ethanol). Found: C, 69·2; H, 9·0; Cl, 9·0. $C_{22}H_{34}ClNO_2$ requires C, 69·5; H, 9·0; Cl, 9·3%.

3-(3-*Cyanopropoxy*)*oestra*-1,3,5(10)-*trien*-17-*one*. A solution of oestrone (2·7 g, 10 mmole) in dimethylformamide (100 ml) containing 5N potassium hydroxide (2 ml, 10 mmole) was heated with γ-chlorobutyronitrile (1·1 g, 11 mmole) at 100° for 1 hr and then stirred at room temperature overnight before pouring into water. The mixture was

TABLE 3. HALOALKYL ETHERS OF OESTRONE.



X	n	Yield (%)	m.p. °C	Crystallising solvent	$[\alpha]_D^\circ$	c	Formula	Found			Required		
								C	H	X	C	H	X
Br	3	46	99–101	methanol	+140	0·97	$C_{21}H_{27}BrO_2$	64·3	6·8	19·8	64·4	7·0	20·4
Cl	4	56	120–122	acetone	+117	1·070	$C_{22}H_{29}ClO_2$	72·6	7·9	—	73·2	8·1	—
Br	5	51	84–86	methanol–ether	+90	0·980	$C_{23}H_{31}BrO_2$	66·2	7·55	18·9	66·0	7·45	19·1
Br	6	26	74·5–76·5	n-hexane	+101	1·035	$C_{24}H_{33}BrO_2$	66·6	7·7	18·6	66·5	7·7	18·4
Br	10	41	66–68	n-hexane*	+88	1·015	$C_{28}H_{41}BrO_2$	68·9	8·7	16·3	68·7	8·4	16·5

* after chromatography.

extracted with ether, and the ethereal extract washed with Claisen's alkali, washed with water until neutral, dried, filtered, and evaporated. Crystallisation of the residue from acetone gave 3-(3-cyanopropoxy)oestra-1,3,5(10)-trien-17-one (1·2 g), m.p. 127–129°, $[\alpha]_D$ + 128° (*c*, 1·025). Found: C, 78·55; H, 8·2%. $C_{22}H_{27}NO_2$ requires C, 78·3; H, 8·1%.

17α-*Allyloestradiol.* Allyl bromide (10 ml) was added at 10° to magnesium (10 g) under anhydrous tetrahydrofuran (100 ml). Once the reaction had started, a solution of oestrone (10 g) in allyl bromide (30 ml) and anhydrous tetrahydrofuran (400 ml) was added dropwise over 2 hr and additional 1 g portions of magnesium were added at half-hourly intervals. Thereafter the temperature of the reaction mixture was allowed to rise to 20° and stirring continued for a further 2 hr. Saturated ammonium chloride solution was then added, the product isolated by extraction with ether, and crystallised from di-isopropyl ether to give 17α-allyloestradiol (10·8 g), m.p. 111–112°, $[\alpha]_D$ + 58° (*c*, 0·98). Found: C, 80·7; H, 8·9. $C_{21}H_{28}O_2$ requires C, 80·7; H, 8·9%.

17α-*Propyloestradiol.* 17α-Allyloestradiol (5 g) was hydrogenated in ethanol (60 ml) over platinum oxide (250 mg), the catalyst filtered off, and the filtrate evaporated to dryness *in vacuo*. The residue was crystallised from methanol to give 17α-propyloestradiol (3·95 g), m.p. 165–167°, $[\alpha]_D$ + 54° (*c*, 0·94). Found: C, 80·3; H, 9·5. $C_{21}H_{30}O_2$ requires C, 80·2; H, 9·6%.

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The relation between heat activation and colony formation for the spores of *Bacillus stearothermophilus*

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Spores suspended in water were heated at 73·5, 100, 115 and 121° and colony counts made. These showed an initial increase followed by an exponential decline. Spores in phosphate buffer showed a reduced activation. Comparison of total (microscopic) and colony counts showed that only about 5% of unheated spores developed into colonies. This increased to about 50% after optimum heat activation.

LITTLE attention has been drawn explicitly to the relationship between total and viable counts of spore suspensions (Valentine & Bradfield, 1954; Bufton, 1959; Powell, 1956). Sublethal heating of spore suspensions of some bacterial species results in an increased viable count (Evans & Curran, 1943; Curran & Evans, 1945; 1947). It is implied from the results of these and other studies (Murrell, 1961) that without preheating only a small proportion of spores of some species developed into colonies under the conditions tested. It is the purpose of this work to study the kinetics of heat activation and kill and to relate total with viable count for aqueous suspensions of *Bacillus stearothermophilus* spores.

Experimental

PREPARATION OF SPORE SUSPENSIONS

Spores of *B. stearothermophilus* NCIB 8919 were obtained from cells grown at 55° on a medium containing Bactotryptone 3 g, Oxoid peptone 6 g, Yeastrel 3 g, Lab-Lemco 1·5 g, agar 25 g. Mn^{2+} 1 ppm, water to 1,000 ml, pH 7 (Kelsey, 1960). The method of Long & Williams (1958) was used to separate vegetative cells from spores which were washed five times using a refrigerated centrifuge. Details of the spore suspension are given in Table 1.

COUNTING METHOD

A spread plate colony count method (Roberts, 1961) was used to screen several counting media, using five replicate plates for each count. These were Oxoid dextrose tryptone agar with and without 0·1% starch; Oxoid tryptone glucose extract agar with 0·1% starch; Oxoid tryptone soya agar with 0·1% starch and 0·5% dextrose; Antibiotic Assay medium with 0·1% starch, pH 6·6.

The latter medium gave the highest counts and was chosen as the basic medium for subsequent experiments. Its composition is based upon that recommended in the B.P. 1958 and is as follows: Peptone (Oxoid) 6 g, tryptone (Oxoid) 4 g, yeast extract (Yeastrel) 3 g, beef extract (Oxoid

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Lab-Lemco) 1·5 g, Dextrose B.P. 1 g, agar 15 g, soluble starch (Analar) 1 g, water to 1,000 ml.

Fig. 1 illustrates the effect of presence or absence of 0·1% starch in the recovery medium on the count of heat-activated spores.

Maximum colony formation occurred within the range 55°–68°. Routine incubation was at 56° for 3 days.

TABLE 1. DETAILS OF SPORE SUSPENSIONS

Suspension	Diluent	Sporulation time (days)	Total count	Counting chamber size (mm)	% forming colonies
TW	Water	2	10^8	0·1	5
TWH	Water	8	$7·8 \times 10^8$	0·1	4·7
TR	Ringers Solution ..	2	$9·8 \times 10^7$	0·1	3·3
TP	Phosphate Buffer pH 7..	2	$2·1 \times 10^8$	0·1	2
E	Water	5	10^8	0·2	1·4
G	Water (Unwashed) ..	5	4×10^8	0·2	1·2

Variability of counts. Nine replicate counts of a spore suspension were made and the results subjected to an Analysis of Variance (Bailey, 1959) which showed that the between counts variance was not significantly greater than that within counts. The coefficient of variation of the means was 6·1% (Brown, 1962).

METHOD OF TESTING HEAT RESISTANCE

Five drops of spore suspension were introduced into each of several 1 ml sterile glass ampoules using a standard dropping pipette (Cook & Yousef, 1953). The ampoules were then sealed and totally immersed in a Townson & Mercer X27* constant temperature bath. Ampoules were removed after measured time intervals, immediately cooled, and the contents diluted and plated out. Thermocouple readings indicated that 100° was reached in less than 30 sec; 115° and 121° in less than 50 sec.

Reproducibility of exposure time/survivor curves. Time/survivor curves typically showed activation followed by an exponential death rate. Duplicate heat resistance experiments were made at 115° and 121° (Figs 2 and 3) and the analysis of Yousef (1954) used to test if the replicate regression lines could be represented by a common regression. The residual variance between replicate regressions was not significantly greater than that within regressions in each case.

Effect of heating spores at different temperatures. Spore suspensions were heated at 73·5°, 100°, 115° and 121° (Figs 4–7).

The highest colony count caused by heat stimulation was estimated from the results shown in Figs 4–7 for suspension TW and the corresponding time noted for each exposure temperature. Time for maximum heat activation was plotted against exposure temperature (Fig. 8). The exposure times given in the figures were calculated from the time the ampoules were immersed in the heating-bath. This was because the lag period was found to be important in stimulating an increase in the viable count at 115° and 121°.

* Townson & Mercer, Ltd., Croydon.

METHOD OF MAKING TOTAL COUNTS

Helber slides were used of stated depth 0·1 mm ± 0·001 mm with improved Neubauer* ruling. The spores in 64 preselected squares were counted using a phase contrast microscope. The experimental details have been described previously (Cook & Lund, 1962; Lund, 1962).

Replicate counts of several spore suspensions were made using five slides in each case and the mean coefficient of variation was calculated to be 5%. Earlier counts using slides of 0·02 mm depth gave a mean coefficient of variation of 16%.

PERCENTAGE OF SPORES FORMING COLONIES

Total and colony counts were made of several suspensions of spores cultured on different occasions and the results used to calculate the percentage of spores forming colonies (Table 1).

Results and discussion

The presence of starch in the recovery medium increased the counts of both heated and unheated spores (Fig. 1). This has been noted by other workers (Olsen & Scott, 1950; Murrell, Olsen & Scott, 1950).

The incubation period necessary for maximum colony formation for all the suspensions tested was inversely proportional to the heating period and to the reaction temperature (Brown, 1962). Curran & Evans (1954; 1947) found that heat activation resulted in the earlier development of colonies. Conversely, heating at temperatures close to lethal temperature has substantially increased the time necessary for incubation of thermophilic anaerobes such as *Clostridium botulinum* and also for *Bacillus* species under certain conditions (Schmidt, 1954). It would seem that the recovery medium used in this work does not contain inhibitors which make an increased incubation time necessary for heated spores.

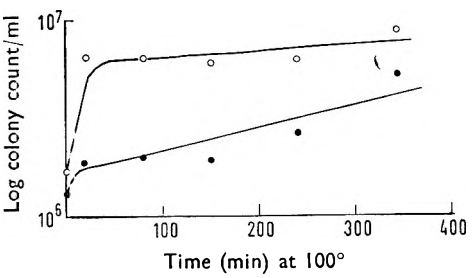

FIG. 1. Effect of presence or absence of starch in the recovery medium for heated *B. stearothermophilus* spores (Suspension E). ○ With starch. ● Without starch.

The heating of suspensions of *B. stearothermophilus* spores in water, but not phosphate buffer, gave characteristic time/survivor curves. The colony count rose to a maximum as exposure time increased after which there was an apparent exponential death rate. No "tail off" was observed.

* Messrs Hawksley and Sons Ltd., 17 New Cavendish Street, London, W.1.

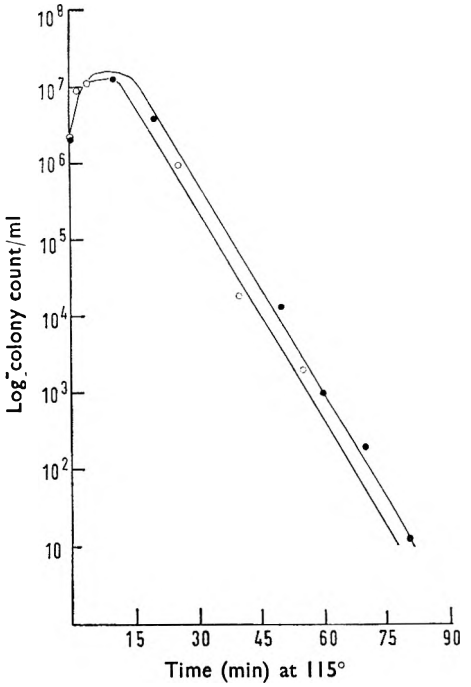

FIG. 2. Replicate heat resistance experiments at 115° with *B. stearothermophilus* spores (Suspension TW). ● Experiment 1. ○ Experiment 2.

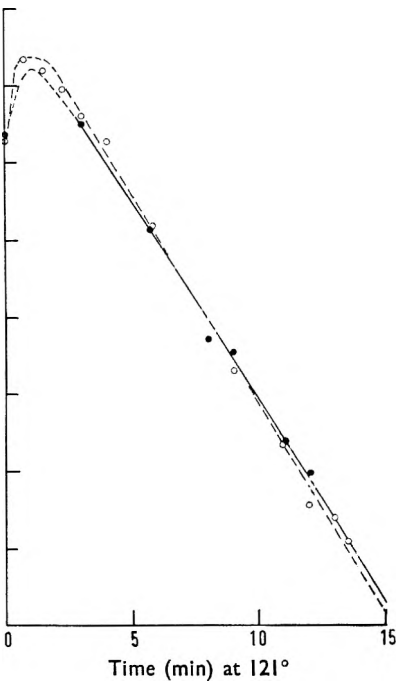

FIG. 3. Replicate heat resistance experiments at 121° with *B. stearothermophilus* spores (Suspension TW). ● Experiment 1. ○ Experiment 2.

Vas & Proszt (1957) obtained similar curves with dilute suspensions of *Bacillus cereus* spores. They found an exponential decline followed by a reduced rate of kill with concentrations exceeding about 10^8/ml. They found evidence suggesting that this "tail off" was due to the presence of a minute and constant fraction (about 1 in 10^7 or 10^8) of very resistant spores.

Amaha & Ordal (1957) found that the logarithm of survivors/time curve for heated spores of *Bacillus coagulans* showed an initial shoulder. They

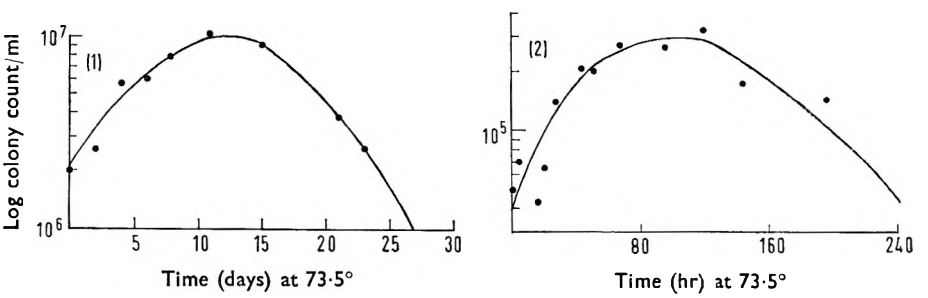

FIG. 4. Effect of heating at 73·5° upon the colony count of *B. stearothermophilus*. Experiment 1, suspension TW. Experiment 2, suspension TP.

made both plate and direct microscopic counts throughout their work and found no significant difference between them. They suggested that the shoulder was due to changes in the resistance of the spores.

The time required to reduce the colony count to unity (single survivor time), for suspension TW at each exposure temperature was calculated from the equation for the exponential part of the curve at 100°, 115° and 121° and there was an exponential relationship between temperature and the calculated single survivor time. This time will depend also upon the initial concentration which was about 2×10^6/ml (Brown, 1962).

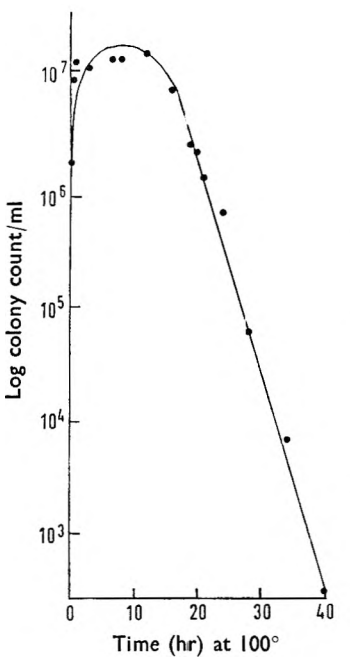

FIG. 5. Effect of heating at 100° upon the colony count of *B. stearothermophilus* (suspension TW).

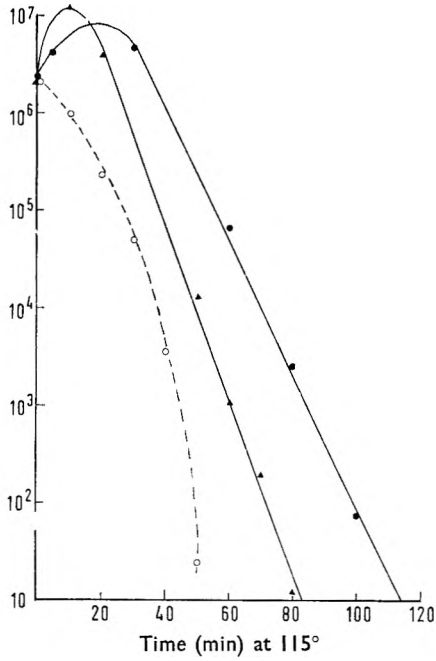

FIG. 6. Effect of heating at 115° upon the colony count of *B. stearothermophilus*. ● Suspension E. ▲ Suspension TW. ○ Suspension TP.

There is a smooth relationship between temperature and logarithm of the exposure time necessary to produce maximum activation (Fig. 8). This graph could be used to predict the approximate time for maximum activation within the range plotted for spores suspended in water. A similar relationship may be calculated from the results given by Murrell (1961, Fig. 1) of log viable count/heating time for spores of *B. coagulans* heated at different temperatures.

Spores in 0·1M phosphate, pH 7 at 115° showed no activation and gave a time/survivor curve concave downwards (TP in Fig. 6).

This latter effect was possibly because at the final exposure times, when counts were low, there was little and eventually no dilution of the spores suspended in phosphate buffer and consequently initiation of germination

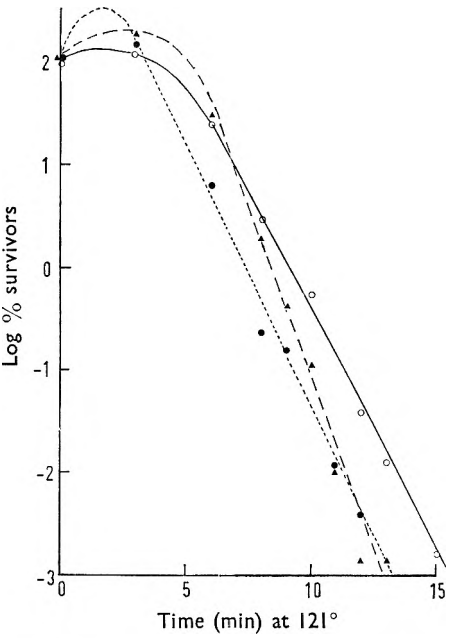

FIG. 7. Effect of heating at 121° upon the colony count of *B. stearothermophilus*. ○ Suspension E. ▲ Suspension TH. ● Suspension TW.

in the recovery medium may have been inhibited by the phosphate (Williams & Hennessee, 1956; Murty & Halvorson, 1957). Spores heated for only a short period were much diluted in distilled water before plating out and the concentration of phosphate in the recovery medium was likely to have been insignificant.

Activation in the presence of 0·1M phosphate did take place at 73·5° although the time for maximum activation of suspension TP was about 4 days. The time for maximum activation for suspensions in water

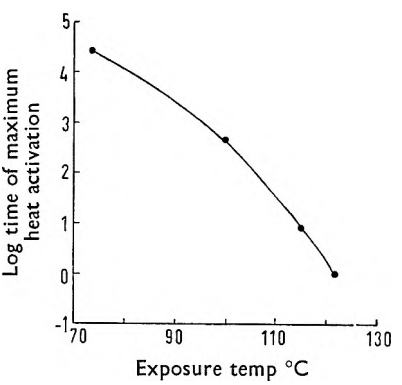

FIG. 8. Relationship between exposure temperature and time for maximum heat activation for *B. stearothermophilus* spores (TW).

(TW) of similar spore concentrations was about 12 days (Fig. 4). The increase in count which occurred in each case was of the same order (6-fold).

The depressant effect of phosphate in the heating medium upon the activation of spores is of particular interest. Gerhardt & Black (1961) have shown that *B. cereus* spores are permeable to phosphate to a considerable extent (40%). It seems possible that if heat activation involves the stimulation of some mechanism connected with initiation of metabolism, then the presence of phosphate within the cell at the time of heating may prevent this stimulation.

It is apparent that the heating up time necessary for the suspension to achieve either 115° or 121° was important for activation. During this period of about 50 sec there was a substantial increase in colony count when compared to that of the unheated control (Figs 2 and 3).

0·02 mm depth counting chambers gave significantly more variable total counts than those obtained with 0·1 mm slides. Norris & Powell (1961) found that total counts with 0·02 mm slides were systematically in excess of the true values by 10–50%.

5% or less of the unheated spores germinated and gave colonies (Table 1). These results indicate that after maximum heat activation (10-fold) about 50% of the spores developed into colonies after subculture.

Microscopic examination of the heat activated spores showed that they retained their refractility under phase contrast illumination. Consequently it seems possible that under other conditions substantially all of the spores may be capable of colony formation. It is interesting to note that a similar low percentage colony formation has been observed with *Rhizopus nigrificans* spores (Brown & Bullock, 1960).

The practice in the literature of quoting only the time necessary for maximum heat activation may be misleading. It would be more informative also to state the percentage of spores which produce colonies.

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Preliminary chemical examination of digitalis tissue cultures for cardenolides

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Three *Digitalis* species were grown as callus or suspension tissue cultures, extracted and examined by paper chromatography. Kedde-positive substances (cardenolide glycosides) were found.

REVIEWS of the potential of higher plant tissue cultures for producing useful compounds (Nickell, 1962; Staba, 1963), and procedures for growing digitalis as static (Staba, 1962) or suspension cultures (Staba & Lamba, 1963) have been published. This communication establishes that callus tissues, and the cells and medium from suspension cultures, of three *Digitalis* species contain secondary plant products similar to digitalis cardiac glycosides.

Material

TISSUE CULTURES

Digitalis lanata Ehrh., *Digitalis purpurea* L., and *Digitalis purpurea* var. *gloxinaeflora* Hort. tissue cultures were grown on our modification (Staba & Lamba, 1963) of Murashige's and Skoog's tobacco medium with (Medium A) and without (Medium B) 2,4-dichlorophenoxyacetic acid (2,4-D) 1×10^{-6}. The tissue cultures were established from germinated seeds, and has been subcultures at three 6-week intervals for approximately 2 years. The callus tissues extracted were approximately 6 weeks old, and the suspension cultures extracted were 3 weeks old. *D. lanata* tissue cultures form organised structures when grown in Medium B, but principally unorganised cell aggregates when grown in medium A (Staba & Lamba, 1963).

Callus tissues were grown in 1-oz. wide-mouth prescription vials (18 ml medium) and cell suspensions in 250 ml Erlenmeyer flasks (50 ml medium) at room temperature (about 27°) and light conditions. Suspension cultures were grown on a reciprocal shaker (88 cycles/min; 9 cm stroke).

Experimental

GENERAL PROCEDURES

Methanol (50%) was used for the initial extraction of callus tissue and suspension cells. The liquid medium from suspension cultures was diluted with methanol to a 50% concentration. Methanolic extracts were purified by lead subacetate addition and a counter current technique for cardenolides (Euw, Hess, Speiser & Reichstein, 1951). The extracts obtained were passed through five separatory funnels, the first containing 3 ml distilled water, the next two containing 3 ml 2N sodium carbonate

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solution, and the last two containing 3 ml distilled water. All partially purified solutions were dried with anhydrous sodium sulphate, evaporated, then dried to a constant weight under reduced pressure (Table 1), and examined chromatographically.

TABLE 1. TISSUE QUANTITIES AND EXTRACTS OBTAINED FROM DIGITALIS TISSUE CULTURES

Tissue culture	Medium and material extracted*	Amount wet cells (g) or medium (ml) extracted	Crude methanolic extract (g)†	Partially purified extracts (mg)			
				Chlorof.	Ether	Chlorof.-Ethanol (7:1)	Chlorof.-Ethanol (2:1)
D. lanata ..	A Ca	208·5	2·97	9·0	—	—	52·0
	A SC	99·5	1·90	45·0	—	25·0	37·0
	A SM	720	—	—	—	34·0	77·0
	B SC	51·5	1·43	42·5	—	27·0	28·5
	B SM	850	—	—	—	91·0	96·0
D. purpurea ..	A Ca	156·7	2·53	11·0	—	—	12·0
	A SC	82·5	1·38	3·0	5·0	—	9·0
	A SM	1·010	—	24·0	19·0	—	43·0
D. purpurea var *gloxinaeflora*..	A Ca	77·3	1·50	8·0	10·0	—	16·0
	A SC	56·0	1·58	4·0	8·0	—	18·0
	A SM	795	—	5·5	9·0	—	27·0

* Ca = Callus Cells, SC = Suspension Cells, SM = Suspension Medium
† Weight of dried material contained in 50 ml methanolic extract

D. lanata EXTRACTION

Callus tissue from agar medium A. The callus tissue from 86 vials (208·5 g wet wt) was homogenised with 210 ml of 50% methanol in a Waring blendor and macerated for two weeks. The cells were filtered on to a weighed filter paper, rinsed with 50% methanol and dried at 105° to a constant dry weight (7·95 g).

The methanolic extract (440 ml) of the callus tissue was diluted to 500 ml with 50% methanol and lead subacetate (10%, 70 ml) added. The resulting precipitate was filtered out and the excess lead in solution precipitated by adding dibasic sodium phosphate (10%). The pH of the solution after filtration was 6·0. The solution was concentrated under reduced pressure at 40° to 25 ml and counter-current extracted with chloroform, 6 × 25 ml, chloroform-ethanol (2:1), 6 × 25 ml, and partially purified as described under General Procedures.

Suspension cultures from liquid medium A. Twenty flasks contained 99·5 g wet wt of cells and 720 ml medium. The distilled water (200 ml) used to wash the cells and flasks was combined with the medium.

The cells (99·5 g) were ground in a mortar with methanol (88%, 150 ml). An additional quantity of methanol (50%) was added and the mixture macerated for 2 weeks. After filtration, 675 ml methanolic solution and 5·11 g (dry wt) extracted cells were obtained. The methanolic solution was purified with lead subacetate and concentrated to 20 ml. This concentrate was extracted with chloroform, 6 × 25 ml, chloroform-ethanol (7:1), 6 × 25 ml, chloroform-ethanol (2:1), 6 × 25 ml, and partially purified as described under General Procedures.

The combined medium-rinse water solution (720–200 ml) was diluted with methanol (920 ml), purified with lead subacetate solution and

concentrated to 85 ml. This concentrate was extracted with chloroform-ethanol (7:1), 5 × 100 ml, chloroform-ethanol (2:1), 5 × 100 ml, and partially purified as described under General Procedures.

Suspension cultures from liquid medium B. Twenty flasks contained 51·5 g (wet wt) cells and 850 ml medium. The distilled water (100 ml) used to wash the cells and flasks was combined with the medium.

The cells (51·5 g) were subjected to the same procedures as described for *D. lanata* cells grown in liquid medium A. After extraction and maceration they yielded 600 ml methanolic solution and 3·47 g (dry wt) extracted cells. The methanolic concentrate obtained (20 ml) was solvent partitioned and treated as previously described. The combined medium-rinse water solution (850–100 ml) was diluted with 950 ml methanol and purified with lead subacetate solution. The methanolic concentrate obtained (77 ml) was solvent extracted and also treated as previously described.

D. purpurea EXTRACTION

Callus tissue from agar medium A. The callus tissue from 61 vials (156·7 g wet wt) was extracted and purified as described for *D. lanata* callus and yielded 600 ml methanolic solution and 4·47 g dry extracted cells. The methanolic concentrate obtained (25 ml) was counter-current extracted and partially purified.

Suspension cultures from liquid medium A. Twenty-five flasks contained 72·5 g (wet wt) cells and 1·01 litres medium.

The cells (82·5 g) were extracted and macerated as described for *D. lanata* cells grown in liquid medium A and yielded 300 ml methanolic solution and 4·48 g (dry wt) extracted cells. The methanolic concentrate (20 ml) was counter-current extracted with ether 6 × 25 ml, chloroform 6 × 25 ml, chloroform-ethanol (2:1) 6 × 25 ml, and partially purified as described under General Procedures.

The medium (1·01 litres) was extracted as described for *D. lanata* cells grown in liquid medium A. The methanolic concentrate (100 ml) was counter-current extracted with ether 5 × 100 ml, chloroform 5 × 100 ml, chloroform-ethanol (2:1) 5 × 100 ml, and partially purified as described under General Procedures.

D. purpurea VAR. *gloxinaeflora* EXTRACTION

Callus tissue from agar medium A. The callus tissue from 23 vials (77·3 g wet wt) was extracted as described similarly for *D. lanata* callus and yielded 400 ml methanolic solution and 2·65 g dry extracted cells. The methanolic concentrate obtained (15 ml) was counter-current extracted and partially purified as described for *D. purpurea* cells.

Suspension cultures from liquid medium A. Twenty flasks contained 56·0 g (wet wt) cells and 795 ml medium.

The cells and medium were purified and extracted as described for *D. purpurea* medium and cells. After extraction and maceration, 600 ml of methanolic solution and 2·82 g of dry extracted cells were obtained.

CHROMATOGRAPHY OF DIGITALIS EXTRACTS

Whatman No. 1 paper chromatograms were developed by the decending technique at 16° for water-containing solvent systems, and at room temperature for formamide-containing solvent systems (Table 2). The

TABLE 2. SOLVENT SYSTEMS, AND RF VALUES OF KEDDE-POSITIVE SUBSTANCES FROM *Digitalis Lanata* PARTIALLY PURIFIED EXTRACTS

Chromatography solvent system	Solvent system reference	Rf Value
Formamide saturated		*Chloroform extract*
1. Xylene-methyl ethyl ketone (1 : 1) ..	Kaiser (1955)	0·18, 0·10, 0·04
2. Benzene-methyl ethyl ketone (1 : 1) ..	,,	0·18, 0·12, 0·04
3. Chloroform-methyl ethyl ketone (1 : 1) ..	,,	0·15, 0·13
(3 : 1) ..		0·31, 0·16
Formamide containing		*Chloroform extract*
4. Tetrahydrofuran-chloroform-formamide (50 : 50 : 6·5)	,,	0·24, 0·20 0·14
5. Chloroform-formamide saturated ..	Schindler & Reichstein (1951)	0·80, 0·75, 0·7, 0·6, 0·48, 0·30, 0·25, 0·20, 0·15, 0·10
Water saturated		*Chlorof.-ethanol extracts (7 : 1) or (2 : 1)*
6. Methyl ethyl ketone	Miystake & others (1957)	0·63, 0·56, 0·41
7. Butanol-toluene (1 : 1)	Schenker & others (1954)	0·85, 0·80, 0·60, 0·50, 0·45, 0·38, 0·28, 0·20, 0·12

papers were impregnated with water (35%) or with formamide (35–40%) before applying the extracts and marker substances. The papers containing water were dried for 15 min, and those containing formamide for 1 hr at 100°, before spraying with Kedde reagent.

Results and discussion

Because of the small tissue quantities available, paper chromatography was used to separate and detect the cardenolides.

Table 2 depicts the results from chromatograms of *D. lanata* tissue culture extracts. The chloroform extracts were best resolved by solvent system 5, and the chloroform-ethanol extracts by solvent system 6. The tissue cultures did not produce lanatosides A, B, or C but did produce other Kedde-positive polar substances. Extracts from medium B contained larger amounts of Kedde-positive substances than extracts from medium A. Also present on chromatograms from medium A were yellow spots which gave a red rather than the usual violet colour with Kedde reagent. Five Kedde-positive (purple reaction) substances were found in *D. purpurea* extracts from callus cells and suspension medium and in *D. purpurea* var *gloxiniaeflora* extracts.

The quantity of Kedde-positive substances in digitalis tissue culture extracts is low as inferred by the quantity of extract applied to chromatograms (0·5–1·0 mg). The detection sensitivity of digitalis glycosides with Kedde reagent on paper chromatograms is approximately 0·01 to 0·05 mg, thus indicating about 0·002% to 0·02% total cardenolides in *D. lanata* grown in liquid medium A. A concentration range of 0·1% to 0·2% lanatosides A, B, and C has been reported for intact plants of *D. lanata* (Ramstad, 1959).

It is concluded that digitalis tissue cultures produce cardenolides different from those in the intact plant, and that these cardenolides are produced in larger amounts from the culture medium lacking 2,4-D than from the medium containing it. Work is in progress to isolate and characterise the cardenolides produced by digitalis tissue cultures.

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Factors influencing the action of glycine on *Escherichia coli* in nutrient broth and in hypertonic medium

A. D. RUSSELL AND R. V. JOHN

The effect of various glycine concentrations in nutrient broth and in a hypertonic nutrient medium against *E. coli* at various temperatures has been examined. Optimum spheroplast formation was found to occur in bacteria which were in the logarithmic growth phase. Glycine has no action on the viability of non-growing bacteria.

IT has been known for many years that glycine exerts an antibacterial effect against *Escherichia coli* (Gordon & McLeod, 1926). More recently, it has been shown that glycine induced lysis of washed suspensions of this organism (Cowles, 1947; Maculla & Cowles, 1948; Gordon, Hall & Stickland, 1951).

Jeynes (1957) found that, in nutrient medium supplemented with high concentrations of sucrose, glycine "removed the cell walls" of certain bacteria to produce protoplasts which were stable in this medium. Jeynes (1961) later produced evidence to substantiate his statement that the spherical bodies produced as a result of glycine treatment did not contain any of the original cell wall constituents. This is in contrast to the findings of Cota-Robles & Duncan (1962) that the osmotically labile spherical forms of *E. coli* B, obtained in the presence of certain D-amino-acids, possess cell wall material.

D-Amino-acids have also been found to induce spheroplasts in *Alcaligenes fecalis* (Lark & Lark, 1959) and in *Rhodospirillum rubrum* (Tuttle & Gest, 1960). However, apart from the discovery (Cota-Robles & Duncan, 1962) that optimum conversion of bacteria to spheroplasts is obtainable only with cells in the early logarithmic phase of growth, little information is available on the factors influencing spheroplast induction. The present investigation has been concerned with a study of the effect of temperature of incubation, age of bacterial cells and glycine concentration on the effect of this amino-acid against *E. coli* in both nutrient broth and a hypertonic nutrient medium.

Experimental and results

MATERIALS

The organism was a laboratory strain of *E. coli* Type I, which has previously been used in this laboratory (John & Russell, 1963). Nutrient broth and nutrient agar were prepared from the respective Oxoid granules. Hypertonic medium consisted of nutrient broth containing 0·25% w/v magnesium sulphate, $MgSO_4,7H_2O$, and either 0·33M or 0·66M sucrose. The pH of all media was 7·4.

Sucrose, glycine, magnesium sulphate, disodium hydrogen phosphate and potassium dihydrogen phosphate, were of analytical reagent quality.

Water was obtained from an all-glass still.

From the Welsh School of Pharmacy, Welsh College of Advanced Technology, Cardiff, Wales.

METHODS

Viable counts were made by serial dilution in sterile water, followed by plating into nutrient agar. Plates were incubated at 37° and colony counts were made after 48 hr.

Measurement of bacterial growth was made with the Spekker absorptiometer, using Standard 20 mm cells, and a standard Kodak filter No. 6. The cells, with specially prepared metal caps, were sterilised by dry heat for 30 min at not above 150°. In all experiments, control media (bacteria absent) were used.

Examination of spheroplasts was made by means of a Cook-Troughton microscope, under oil-immersion, × 100 objective, and by phase contrast.

Effect of glycine on E. coli *at various temperatures.* In this series of experiments, 0·2 ml of an overnight 37° broth culture of *E. coli* was added to 20 ml of nutrient broth or of hypertonic medium, containing varying concentrations of glycine. Incubation was carried out at the required temperature, and extinction measurements made at intervals.

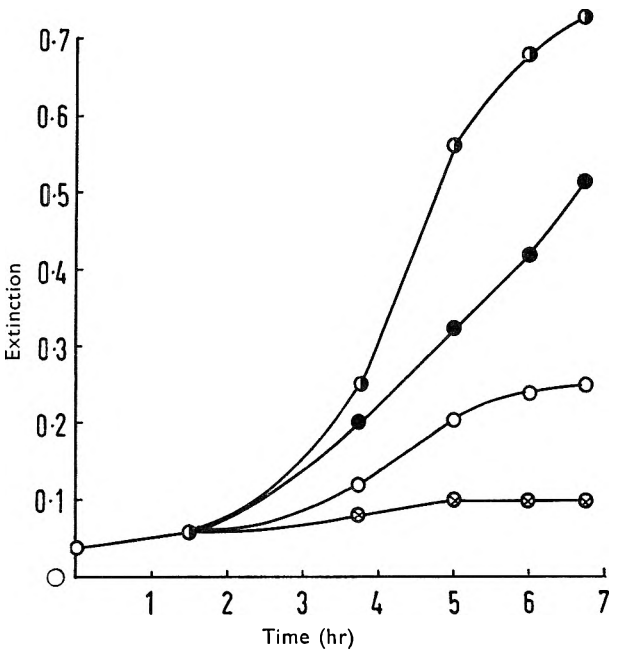

FIG. 1. Effect of glycine on *E. coli* in nutrient broth containing 0·33 M sucrose and 0·25% w/v $MgSO_4,7H_2O$ at 37°. ◑—◑ Glycine absent. ●—● Glycine 10 mg/ml. ○—○ Glycine 15 mg/ml. ⊗—⊗ Glycine 20 mg/ml.

Typical examples of the effect of glycine on *E. coli* are provided in Figs 1 and 2. Results of the other experiments can be summarised as follows: Extinction at 44° showed the greatest increase with time when glycine was absent, and the least when the highest concentration of glycine (15 mg/ml) was present. Extinction was greater when 0·33M sucrose was included than with 0·66M sucrose. At 20°, extinction

increased slowly after a 2 hr lag phase in 0·33M sucrose Mg^{2+} broth (glycine absent) and to a still slower extent in the presence of 10 mg/ml glycine. With glycine absent, a 6 hr lag phase was apparent in 0·66M sucrose Mg^{2+} broth; no increase in opacity was evident even after 24 hr when 10/ml glycine was included.

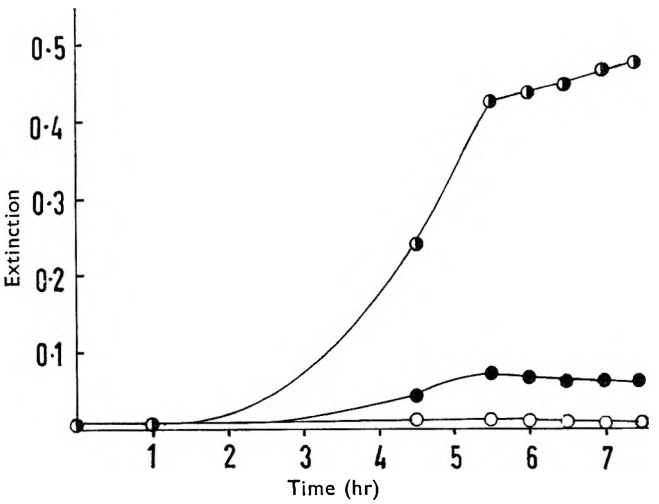

FIG. 2. Effect of glycine on *E. coli* in nutrient broth containing 0·66 M sucrose and 0·25% w/v $MgSO_4,7H_2O$ at 37°. Key: as in Fig 1.

To determine whether an increase in extinction when the hypertonic medium was employed could be correlated with spheroplast formation, samples were removed and examined microscopically (Table 1).

Effect of glycine on the viability of washed suspensions of E. coli. An overnight 100 ml 37° broth culture of the organism was centrifuged, the residue washed twice with 40 ml of sterile water, and finally resuspended in 12 ml of sterile water. From this, 3 × 3 ml volumes were reserved, and made up to 20 ml with buffer solution and glycine to give final concentrations of glycine of 0, 10 and 20 mg/ml. The pH in each case was 7·3. Opacity measurements during subsequent incubation at 37° gave erratic results; however, the fact that there is no loss in viability after treatment for 5 hr (Table 2) indicates that the organisms have not suffered lysis during this period.

Effect of glycine on the viability of E. coli *at* 4°. The non-lethal effect of glycine against "resting" cells of *E. coli* was confirmed by treatment of the organism with the amino-acid in 0·33M sucrose-Mg^{2+}-broth at 4° (Table 3). Spheroplasts were not induced by any glycine concentration.

Effect of glycine on logarithmic phase of E. coli Glycine induces spheroplast formation at growth temperatures but not at 4°, and has no lethal effect on washed suspensions (Table 2). This points to the action of this substance being directed, at least in part, towards an inhibition of cell wall synthesis. This being so, it would be expected that the

TABLE 1. MICROSCOPICAL EXAMINATION OF THE EFFECT OF GLYCINE ON *E. coli* AT VARIOUS TEMPERATURES

Nutrient broth containing 0·25% Mg^{2+} and	Concn. of glycine* (mg/ml)	Temp. of incubation (°C)	Microscopical examination at 5 hr
0·33M sucrose	10, 15 and 20	4	No spheroplasts induced
	10	20	Spheroplasts; small
	15		Optimum number of spheroplasts; small
	20		Spheroplasts; small
	10	37	Morphological variants
	15		Spheroplasts
	20		Optimum number of spheroplasts
	10	44	Spheroplasts
	15		Spheroplasts
	20		Optimum number of spheroplasts†
0·66M sucrose	10, 15 and 20	4	No spheroplasts induced
	10, 15 and 20	20	Spheroplasts poorly developed
	10	37	Optimum number of spheroplasts
	15		Few spheroplasts
	20		No spheroplasts observed
	10	44	Few spheroplasts at 5 hr
	15		(Small, numerous spheroplasts
	20		after 24 hr)

* No spheroplasts formed in the absence of glycine.
† Variety of spheroplasts forms, including "crescents," observed.

antibacterial, and hence the spheroplasting, activity of glycine would be enhanced if tested against young, logarithmic phase bacteria as opposed to non-growing or stationary phase cells.

TABLE 2. EFFECT OF GLYCINE ON WASHED SUSPENSIONS OF *E. coli* AT pH 7·3

Glycine conc. (mg/ml)	Viable count/ml at 0 hr	Viable count/ml after 5 hr at 37°
0	$1·4 \times 10^8$	$1·4 \times 10^8$
10	$1·4 \times 10^8$	$1·5 \times 10^8$
20	$1·4 \times 10^8$	$1·5 \times 10^8$

A 10 ml overnight 37° broth culture of *E. coli* was centrifuged, and the residue washed twice with 2 ml of sterile water, and finally resuspended in 1 ml of broth. This suspension was added to 19 ml of broth in a mixing tube and the mixture then transferred to a Spekker cell which was incubated at 37°. Readings against a control nutrient broth (bacteria absent) were made at intervals, and when it was observed that the organism had entered the logarithmic phase of growth, 1 ml samples were removed

TABLE 3. EFFECT OF GLYCINE ON *E. coli* AT 4°

Glycine conc. (mg/ml)	Viable count/ml at 0 hr	Viable count/ml after 5 hr
0	$4·6 \times 10^6$	$4·6 \times 10^6$
10	$4·6 \times 10^6$	$3·9 \times 10^6$
15	$4·6 \times 10^6$	$3·7 \times 10^6$
20	$4·6 \times 10^6$	$4·8 \times 10^6$

and added to 0·33M sucrose - Mg^{2+} - broth containing final concentrations of glycine of 0, 10, 15 and 20 mg/ml. The final volume in each case was 20 ml. Incubation was carried out at 37°, and extinction measurements made at intervals (Fig. 3).

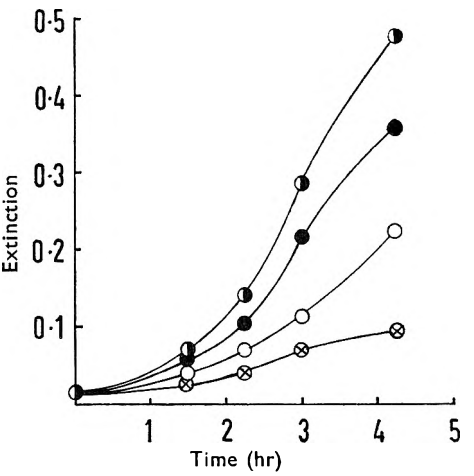

FIG. 3. Effect of glycine on logarithmic phase *E. coli* in nutrient broth containing 0·33 M sucrose and 0·25% w/v $MgSO_4,7H_2O$ at 37°. Key: as in Fig. 1.

Microscopical examination revealed that spheroplasts induced by this method were larger (7·5–9·5 μ) than those induced in the earlier experiments (4–4·5 μ average).

Discussion

The finding that glycine induces spheroplast formation (McQuillen, 1960) has been amply confirmed in the present investigation. The rate at which multiplication is taking place at the time of treatment with glycine is important, since optimum conversion of rods to spheroplasts occurred when the amino-acid was added to logarithmic phase cells (Fig. 3, Table 1). Further, since the spheroplasts had an average diameter much greater than that of those induced from stationary phase cultures, it can be inferred that the spheroplasts were probably produced at an earlier period in the former instance. Additional evidence that glycine is most active against rapidly-growing bacteria is obtained from the data obtained on spheroplast formation at different temperatures (results described briefly in the text; also Figs 1 and 2, and Table 1). Spheroplasts are best induced at the optimum temperature for growth (Table 1).

Glycine has no effect on non-growing bacteria, since (a) it does not kill *E. coli* at 4° (Table 3), and does not induce spheroplasts at this temperature, and (b) the highest concentration of glycine used, 20 mg/ml, has no effect on the viability of washed suspensions during treatment of the bacteria for 5 hr at 37° (Table 2). This latter finding is in contrast to the results obtained by Gordon and colleagues (Gordon & McLeod, 1926;

Gordon & Gordon, 1947; Gordon & others (1951) and by Maculla & Cowles (1948). Both groups of investigators measured the amount of protein released into the supernatant fluid after the glycine-treated cells had been removed by centrifugation. The period of contact of the amino-acid and bacteria was of longer duration (ca. 16 hr at 37°) and the glycine concentrations were higher, than employed in the present work.

After formation, spheroplasts continue to increase in size, with a consequent increased opacity (Hugo & Russell, 1960). However, although a change in opacity in hypertonic medium is a rapid and convenient method of following spheroplast induction, it should always be accompanied by microscopical examination, since an increased extinction could also be caused as a result of (i) ordinary bacterial multiplication or (ii) the induction of aberrant forms of the type described by Hugo & Russell (1961) in a study of the effect of penicillin on *Aerobacter cloacae*. Spheroplasts were stable in media containing either 0·33M or 0·66M sucrose. No spherical forms were observed in ordinary nutrient broth containing glycine.

Osmotically stable rod forms, which, after removal of spheroplasts by dilution into water, gave rise to colony formation when plated into nutrient agar (John & Russell, 1963) were frequently encountered.

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Activity of eledoisin, other polypeptides and ergometrine on the uterus *in situ* of rabbit and other animal species

G. B. FREGNAN AND A. H. GLÄSSER

The oxytocic activity of eledoisin on the uterus *in situ* of different animal species has been studied and compared with other oxytocic drugs. Eledoisin stimulated the rabbit uterus at doses as low as 0·01–0·1 μg/kg i.v. but had no significant oxytocic action on the uterus of cat, rat, and guinea-pig. It is, however, more active on the vascular than on the non-vascular smooth muscle preparations of the same animal species. The oxytocic activity of oxytocin and ergometrine, on the other hand, has been confirmed on almost all the animal species studied in doses which did not significantly alter the blood pressure (at least in the rabbit).

In the rabbit the oxytocic effect of ergometrine but not that of eledoisin and oxytocin was abolished by the adrenolytic drugs dibenzyline, piperoxan and hydergine. Hypertensin also stimulated almost all the uteri tested, whilst bradykinin was not active in the doses used.

THE endecapeptide eledoisin has a potent action on vascular and non-vascular smooth muscle organs. In the cardio-vascular system, it dilates the vascular bed to cause hypotension particularly in the dog (Erspamer & Anastasi, 1962; Olmsted & Page, 1962; Erspamer & Glässer, 1963; Bergamaschi & Glässer, 1963) and in man (Sicuteri, Fanciullacci, Franchi & Michelacci, 1962; Shapiro, Kontos, Page Manck & Patterson, 1963). A pressor effect was observed in the chicken and to a lesser extent in the rat (Erspamer & Glässer, 1963). Both *in vivo* and *in vitro*, eledoisin possesses an intense stimulating action on non-vascular smooth muscle preparations including the isolated uterus preparations from rabbit, rat, guinea-pig, cat, and dog (Erspamer & Falconieri-Erspamer, 1962). Recently, Stürmer & Berde (1963) confirmed the results described above and showed an oxytocic action of eledoisin on the rabbit uterus *in situ*

Eledoisin (Erspamer & Glässer, 1963) and ergometrine (Konzett, 1960) produce responses on the chicken blood pressure similar to those seen with sympathomimetic drugs. Also, the oxytocic action of ergometrine on the rabbit uterus *in situ* has been shown by Konzett (1960) to resemble sympathomimetics. It therefore seemed interesting to investigate further the mechanism of action of eledoisin and other polypeptides on the uterus *in situ* of the rabbit and other animal species.

Material and methods

Drugs. Synthetic eledoisin (prepared by Sandoz and Farmitalia); synthetic oxytocin (Syntocinon); synthetic bradykinin; 1 methyl-D-lysergic acid butanolamide (UML 491); a mixture containing equal parts of dihydroergocornine, dihydroergocristine and dihydroergocryptine(Hydergine); synthetic hypertensin; (–)-adrenaline; 933 F (piperoxan); (–)-noradrenaline; ergometrine; 5-hydroxytryptamine (5-HT); acetylcholine;

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mepyramine; hexamethonium; atropine; histamine; dibenzyline. Drugs were administered intravenously. Blood pressure was recorded from the carotid artery using a mercury manometer. Uterine activity *in situ* was recorded kymographically using an isotonic gravity lever attached to one uterine horn following Rothlin's procedure (1938) modified as described below.

Rabbit, guinea-pig, and cat uterus. The ovarian and cervical ends of a uterine horn were attached to a rod, and the movements recorded from a thread attached to the centre of the uterus. With pregnant animals, the foeti were first removed by surgery before commencement of the recording. Uteri from virgin, non-virgin–non-gravid, gravid, and post-partum animals were used.

Rat uterus. The two uterine horns from ovariectomised young albino rats were dissected free from the ovarian and mesenteric attachments, without disturbing the blood supply, and the combined movements recorded by the method described above.

In all instances the horns were kept in a humid environment. Some virgin guinea-pigs and all of the rats were injected subcutaneously with 1 mg/kg of oestradiol 3–4 days before the experiment. Rabbits and guinea-pigs were anaesthetised with urethane (1 g/kg intravenously to rabbits and intraperitoneally to guinea-pigs); cats were anaesthetised with a mixture of chloralose (60 mg/kg) and urethane (250 mg/kg) intravenously after pre-anaesthesia with ether. Some rats were anaesthetised with amylobarbitone sodium (90 mg/kg i.p.) others were anaethestised with ether, pithed and then maintained by artificial respiration.

Results

RABBIT BLOOD PRESSURE

Eledoisin lowered the blood pressure at doses ranging from 0·001–0·01 μg/kg. Oxytocin did not significantly affect the blood pressure at least in the doses tested (0·01–0·5 IU/kg). Ergometrine, at 100 μg/kg and higher, had an irregular action, either lowering or increasing the blood pressure.

RABBIT UTERUS

Most experiments were on the non-virgin–non-gravid uterus which either had a rhythmic activity of low intensity or was completely quiescent.

Threshold doses of eledoisin, 0·01–0·1 μg/kg, elicited spontaneous activity in quiescent uterine muscle and/or induced a small increase in frequency and amplitude of spontaneous rhythmic contractions. Larger doses, 0·2–1 μg/kg, produced powerful contractions of greater amplitude accompanied by a progressive increase in tone and lasting up to 20 min in some instances. This phase of contraction was followed by a quiescent period. The response of the uterus increased with increasing dose (Figs 1 and 2) and no evidence of tachyphylaxis was observed. The effect of eledoisin did not seem to depend on the hormonal state of the uterus because it was also present in virgin, pregnant, and post-partum rabbits.

Threshold doses of oxytocin, 0·01–0·02 IU/kg, produced similar responses on the rabbit uterus and within certain limits the activity increased with the dose (Fig. 1). The responses of the uterus to oxytocin resembled in some ways those seen with eledoisin, but with increasing doses of oxytocin the period of latency decreased and the effect was more evident on the tone than on the amplitude of the contractions. The effect of 0·5 IU/kg of oxytocin lasted for 30 min or more.

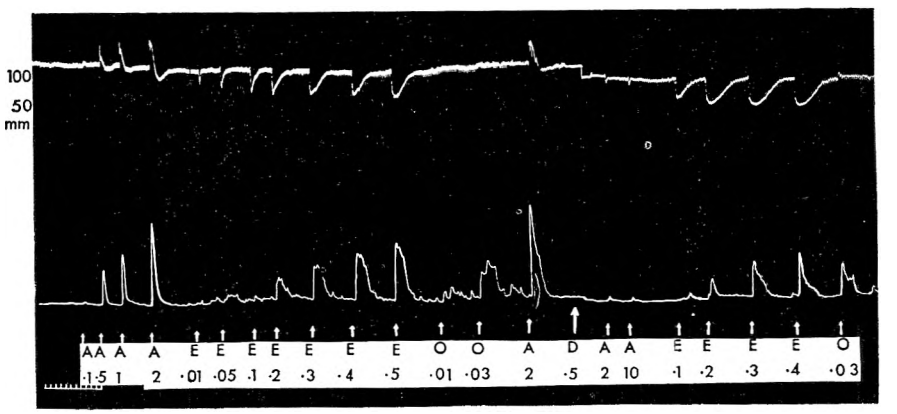

FIG. 1. Rabbit uterus *in situ* and carotid blood pressure in mm Hg. Oxytocic activity of adrenaline (A), eledoisin (E), and oxytocin (O) before and after treatment with 0·5 mg/kg of dibenzyline (D). The numbers denote doses in μg/kg for A and E, IU/kg for O. Time in min.

Hypertensin, 0·1–0·5 μg/kg, also produced contractions of the uterus but was less potent than either eledoisin or oxytocin; larger doses, 5–10 μg/kg, caused an immediate onset of strong contractions of short duration (4–5 min).

Bradykinin, on the other hand did not show any activity even with the highest doses used (10 μg/kg).

Adrenaline and noradrenaline produced a single, prompt, strong contraction on the non-virgin–non-gravid rabbit uterus. The sensitivity in the range of doses used, 0·5–10 μg/kg, appears to be similar for both amines, although a period of relaxation and reduced frequency of spontaneous contractions was sometimes seen after adrenaline. Histamine and 5-HT showed little contractile activity even with the largest doses used (50–100 μg/kg) (Fig. 2).

With ergometrine, our results confirmed the experimental data obtained by Brown & Dale (1935) and Rothlin (1938). Ergometrine, 100 μg/kg evoked rhythmic and powerful contractions; in some experiments the threshold dose was as little as 10 μg/kg.

Antagonism by adrenolytic and other drugs. Adrenolytic drugs, such as hydergine, piperoxan and dibenzyline, administered intravenously in doses able to fully antagonise the responses to adrenaline and noradrenaline on the uterus and on the blood pressure, also inhibited the oxytocic effect of ergometrine, but the responses to eledoisin, oxytocin, and hypertensin

were unaltered. Hydergine, 1 mg/kg, and piperoxan, 10 mg/kg, completely antagonised the uterine contactions to doses of adrenaline, noradrenaline and ergometrine in doses at least five times greater than their

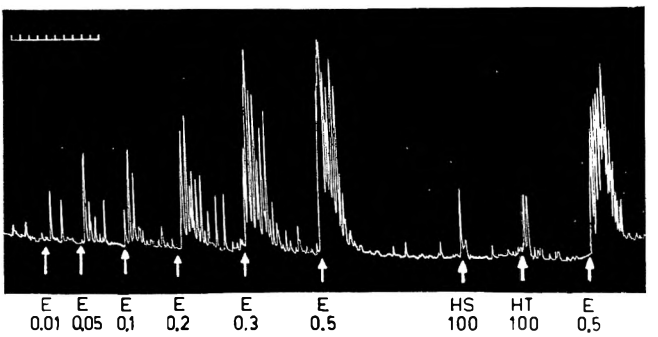

FIG. 2. Rabbit uterus *in situ*. Oxytocic activity of eledoisin (E), histamine (HS), and 5-hydroxytryptamine (HT). The numbers denote doses in μg/kg. Time in min.

minimal threshold doses. Neither adrenolytic drug impaired the responses to eledoisin, oxytocin and hypertensin (Fig. 3). Dibenzyline, 0·5–2 mg/kg, infused intravenously over at least 1 hr, completely abolished the oxytocic response to adrenaline, noradrenaline and ergometrine in doses 10–20 times greater than their minimal threshold doses (Fig. 1), and the responses

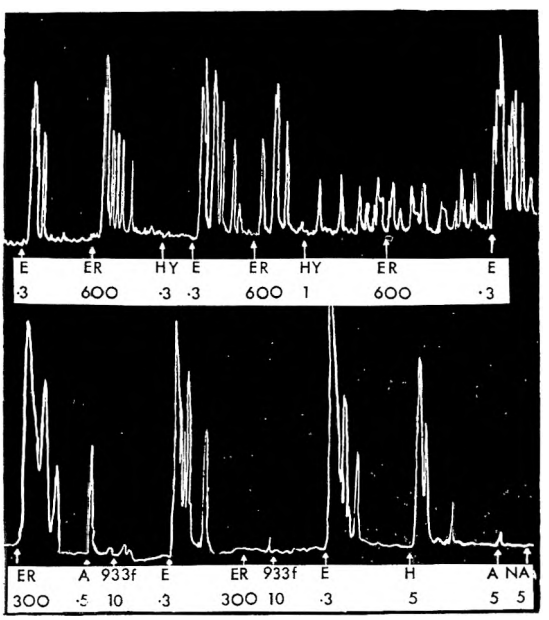

FIG. 3. Rabbit uterus *in situ*. Oxytocic activity of eledoisin (E), ergometrine (ER), hypertensin (H), adrenaline (A), and noradrenaline (NA). Hydergine (HY) in the dose range 0·1–1 mg/kg (mg = ml) and piperoxan (933 f) (10mg/kg) were able to antagonize the oxytocic responses to E, H, and oxytocin (which does not appear on the figure). The figures denote doses in μg/kg. Time in min.

to eledoisin, oxytocin and hypertensin were unaffected. In a few experiments (Fig. 1), after dibenzyline there was a transient reduction (no more than 50%) in the contractions elicited by eledoisin, oxytocin and hypertensin, but the responses to the three polypeptides were restored to the initial value within 2 hr, whilst adrenaline, 10 μg/kg, had no action on the uterus.

Hexamethonium, 15 mg/kg, and atropine, 6 mg/kg, did not impair the responses to threshold and larger doses of eledoisin, oxytocin, hypertensin, ergometrine and catecholamines. Moreover eledoisin was not influenced by pretreatment of animals with mepyramine, 1 mg/kg, and with 1 methyl-D-lysergic acid butanolamide, 1 mg/kg.

RAT UTERUS

The uteri of ovariectomized and oestradiol-pretreated rats were either quiescent or showed small rhythmic contractions. The preparation was not very sensitive to eledoisin; and large doses of the drug, 0·5–1 μg/kg, caused a single and transient contraction. Larger doses did not produce an appreciable increase in the response and tachyphylaxis was sometimes observed.

Oxytocin at the threshold dose, 0·005 IU/kg, produced either persistent rhythmic and powerful contractions in a normally quiescent uterus or else greatly reinforced the spontaneously occurring movements. Large doses, 0·01–0·02 IU/kg, gave a definite rise of the tone which lasted for several hours.

Hypertensin, 0·5–10 μg/kg, produced strong contractions of rat uterine muscle; whilst bradykinin in very large doses, 20–100 μg/kg, gave small and evanescent responses.

Ergometrine, 100–500 μg/kg, caused the prompt appearance of powerful contractions with a rise in tone which lasted for 30–40 min.

GUINEA-PIG UTERUS

Non-virgin–non-pregnant, pregnant, post-partum and oestradiol-pretreated uteri were used. They were either quiescent or showed some spontaneous rhythm.

Eledoisin, 0·01–2 μg/kg, did not elicit any contraction in most experiments. In a few instances, particularly with oestradiol pretreated animals, small and transient contractions of the uterus were seen (Fig. 4).

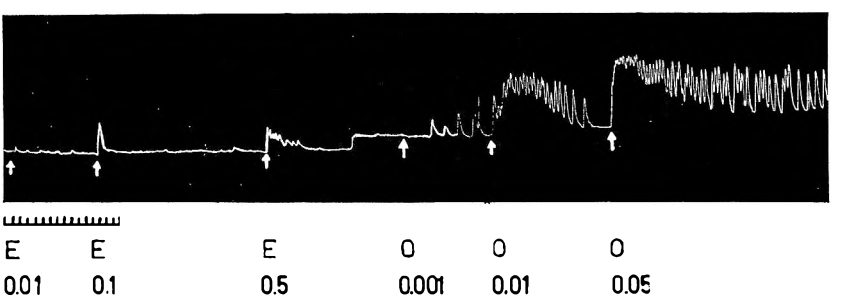

FIG. 4. Guinea-pig uterus *in situ*. Oxytocic activity of eledoisin (E) and oxytocin (O). The numbers denote doses in μg/kg for E and IU for O. Time in min.

The guinea-pig uterus in all the physiological conditions tested was extremely sensitive to oxytocin; the threshold dose being as low as 0·001–0·005 IU/kg. Oxytocin increased either the spontaneous movements, or caused the appearance of numerous powerful rhythmic contractions with an increase of the basal tone between contractions. These lasted for several hours when doses as large as 0·1–0·4 IU/kg were injected.

Hypertensin, 0·05–5 μg/kg, on the pregnant uterus, contracted the uterus in the late stages of pregnancy, whilst at 20 μg/kg it was unable to contract the uterus in the early stages of pregnancy.

Bradykinin, 0·1–5 μg/kg, was inactive in the doses tested; whilst ergometrine was active at the same doses used by Brown & Dale (1935).

CAT UTERUS

Most experiments were made on uteri of cats during the last stage of gestation or few days post-partum. The organ had a naturally high tone with irregular movements and for this reason, the test was more useful in demonstrating the activity of drugs which cause relaxation (i.e., adrenaline) than for oxytocic drugs. In these conditions eledoisin, 1–5 μg/kg, oxytocin, 0·1–0·3 IU/kg, and ergometrine, 200–500 μg/kg, produced an increase in the basal tone but had little effect on the already maximal contractions. Neither bradykinin, 1–10 μg/kg, nor noradrenaline, 1–10 μg/kg, had any action on the uterine smooth muscle; adrenaline 1–40 μg/kg, relaxed the uterus. Hypertensin was not tested.

A few experiments were made on cats in the first period of gestation, in which phase the spontaneous contractions were small and rapid. Neither eledoisin, 0·4–2 μg/kg, nor oxytocin, 0·01–0·1 IU/kg, were very effective on these organs. On the other hand, the responsiveness of the uteri to hypertensin, adrenaline and noradrenaline was good, 1–2 μg/kg of each drug caused the prompt appearance of a single strong contraction.

Discussion

Eledoisin stimulates particularly the rabbit uterus *in situ*, and the response of the uterus is satsifactorily proportional to the dose, 0·01–1 μg/kg (Fig. 1). The uterus *in situ* of rat, guinea-pig and cat is almost insensitive to eledoisin. These results are in accordance with those obtained *in vitro* by Erspamer & Falconieri-Erspamer (1962) and those obtained *in vivo* on the rabbit and cat uterus by Stürmer & Berde (1963). Adrenolytic drugs (dibenzyline, piperoxan, hydergine), in doses which completely abolish the activity of adrenaline and noradrenaline, do not significantly reduce the oxytocic responses of the rabbit uterus to eledoisin. Neither hexamethonium, mepyramine, 1-methyl-D-lysergic acid butanolamide, and atropine modify the responses of eledoisin on the rabbit uterus.

Oxytocin behaves like eledoisin, in that its oxytocic action is unaffected by the antagonists described, but differs from eledoisin in that it contracted the uteri tested from all species.

The mechanism of the responses to ergometrine seems to be different

from that of eledoisin and oxytocin. The three adrenolytic drugs used abolished the contractions of the uterus to ergometrine, as well as to sympathomimetic drugs. This is in accordance with results of Konzett (1960) showing that dibenamine and phentolamine (as well as hydrogenated ergot alkaloids) specifically inhibit the action of ergometrine.

Bradykinin, 5-HT, and histamine appear to be almost inactive on the rabbit uterus. Stürmer & Berde (1963) also found that only very large doses of bradykinin, 44 μg/kg, caused contractions. Hypertensin, on the contrary, is active in almost all the uteri of species tested, on the rabbit however the drug is less active than eledoisin. The adrenolytic drugs and the ganglionic-blocking agents tested do not alter the oxytocic responses of hypertensin on rabbit uterus.

In conclusion, these findings suggest that ergometrine acts at least on the rabbit uterus through an adrenergic mechanism whilst eledoisin and oxytocin (as well hypertensin) act independently. Studies are in progress to elucidate further the mechanism of the oxytocic action of ergometrine in other animal species. Erspamer & Falconieri-Erspamer (1962) and Erspamer & Glässer (1963) and the present results clearly show that eledoisin is much more active on the vascular beds than on the extravascular smooth muscles of the same animal species.

Acknowledgements. We are grateful to Sandoz Ltd., Basel, Switzerland for supplying UML 491 and bradykinin.

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J. Pharm. Pharmacol., 1964, **16**, 751–758 Received May 4, 1964

Effect of chlorhexidine diacetate on "protoplasts" and spheroplasts of *Escherichia coli*, protoplasts of *Bacillus megaterium* and the Gram staining reaction of *Staphylococcus aureus*

W. B. HUGO AND A. R. LONGWORTH

Chlorhexidine prevents the transformation of *Bacillus megaterium* cells to protoplasts by lysozyme, of *E. coli* cells to spheroplasts by penicillin and causes lysis of "protoplasts" and spheroplasts of *E. coli* stabilised in hypertonic sucrose solution. Transformation of *Staphylococcus aureus* cells to the Gram-negative condition occurred in contact with 10 to 800 μg/ml of chlorhexidine and 10, 50 and 100 μg/ml of cetyltrimethylammonium bromide but higher concentrations of the latter prevented this change. Results indicate that chlorhexidine damages the permeability barrier of bacterial cells.

THE adsorption of chlorhexidine by suspensions of *Escherichia coli* and *Staphylococcus aureus* and the leakage and turbidity changes caused by the drug have been described previously and chlorhexidine was thought to owe its bactericidal activity to a physical disorganisation of the permeability barriers of the cell (Hugo & Longworth, 1964).

To examine this hypothesis experiments were made on bacterial forms deprived of some or all of their cell walls.

Definitions. The following terms are used (McQuillen, 1960a): The term protoplast is reserved for the structure derived from a vegetative cell by removal of the cell wall. Where the cell wall is modified to render the organism spherical but it is not known whether the cell wall is entirely absent the term "protoplast" is used. The globular forms produced by growth in penicillin are termed spheroplasts. The feature common to the three forms is a deficiency of a component of the cell wall responsible for structural rigidity so these forms show greater susceptibility to lysis but can be stabilised by suspension in a solution of a non-permeable solute at sufficient concentration to balance the pressure within the depleted cell, for example with sucrose.

Experimental and results

Organisms used in the work were *Escherichia coli* (formerly NCTC 5934), *Bacillus megaterium* NCTC 6005 and *Staphylococcus aureus* (Oxford strain).

Culture media were prepared from Oxoid materials. Nutrient broth consisted of Lab Lemco 1 g, peptone 1 g, sodium chloride 0·5 g, distilled water to 100 ml. When a solid medium was required 1·8% of agar No. 3 was included. Liquid conversion medium for spheroplast preparation was the nutrient broth plus 0·25% w/v $MgSO_4.7H_2O$ and 0·33M sucrose. In all cases the pH after adjustment and sterilisation was 7·3.

From the Department of Pharmacy, Nottingham University.

Chlorhexidine diacetate (I.C.I.) and cetyltrimethylammonium bromide, CTAB (B.D.H.), were obtained commercially. Egg white lysozyme (L. Light & Co. Ltd.) was used throughout.

The bacterial suspensions were prepared by washing 18 hr cultures grown at 37° for *E. coli* and *Staph. aureus* and 30° for *B. megaterium* from the surface of agar, centrifuging at 1,000 *g* for 2 min to remove agar and large clumps, and washing twice with water before resuspension and nephelometric standardisation.

The extinction was determined at a wavelength of 500 mμ in a Unicam SP500 spectrophotometer at 20° ± 1° using 1 cm silica cells against a reference cell containing water. For one experiment an E.E.L. nephelometer was used.

Microscopic examinations were made with a Baker interference microscope and for total counts a Helber counting chamber, 0·02 mm deep, was used. Experiments were in duplicate and representative results are shown.

PREPARATION OF "PROTOPLASTS" OF *E. coli*.

Repaske (1956) discovered that *E. coli* is sensitive to lysozyme in 2-amino-2-hydroxymethylpropane-1,3-diol(tris) buffer in the presence of ethylenediaminetetra-acetic acid (EDTA) and later Repaske (1958) pointed out that the relative amounts and the order of addition of the compounds are also critical. Mahler & Fraser (1956) showed that with *E. coli*, lysis could be prevented if sucrose 0·5M was present when spherical "protoplasts" were produced; lysis occurred upon dilution of the stabilising solution.

Preliminary experiments showed optimal conditions to be when 1 ml of an aqueous suspension of *E. coli* containing 50×10^8 cells/ml was added to 9 ml of the lytic system such that the final suspension contained 0·033M tris buffer pH 7·3 10 μg/ml lysozyme and 200 μg/ml EDTA, Lysis at 20° as estimated by spectrophotometric analysis, was complete in 30 min. Using the conditions outlined above and including 0·5M sucrose in the medium, small spherical bodies were evident on microscopical examination and the conversion appeared to be complete within 30 min of inoculation.

EFFECT OF CHLORHEXIDINE ON "PROTOPLASTS" OF *E. coli*

2 ml of a suspension of *E. coli* containing 50×10^9 cells/ml was added to 198 ml of the Tris/EDTA/lysozyme/sucrose system previously described. After 30 min contact at 20°, 9 ml quantities were removed and added to 1 ml quantities of chlorhexidine in 0·5M sucrose and changes in extinction over a period of time estimated. A control experiment with "protoplasts" added to 0·5M sucrose solution was also made. After 1 hr contact with chlorhexidine, samples were removed and examined by interference microscopy. The extinction determinations are presented in Fig. 1.

Low concentrations of chlorhexidine 5–20 μg/ml caused a rapid decrease in extinction (indicative of lysis) and over this range of

concentrations microscopic examination showed that no "protoplasts" were present but much lysed matter was evident. Higher concentrations of chlorhexidine 40–100 μg/ml produced effects ranging from a slight reduction to an increase in extinction as compared to "protoplasts" suspended in buffer. Microscopic examination showed that whilst no true spherical forms were present there was little evidence of lysis.

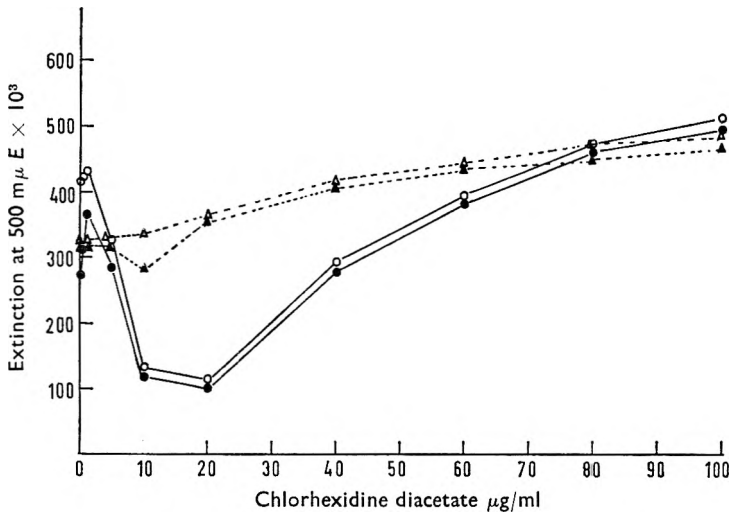

FIG. 1. Effect of concentration of chlorhexidine diacetate on the extinction ($E \times 10^3$ at 500 mμ) of "protoplasts" of *E. coli* in 0·5M sucrose. O—O Extinction after 5 min contact, ●—● extinction after 1 hr contact; and on the extinction of *E. coli* cells suspended in tris buffer pH 7·3. △ - - - △ Extinction after 5 min contact, ▲ - - - ▲ extinction after 3 hr contact.

Fig. 1 also shows the extinction of an *E. coli* cell suspension 50×10^7 cells/ml in 0·033M tris buffer read against a reference cell containing water after 5 min and 180 min contact at 20°. The slight decrease in extinction which occurred after 180 min over the concentration range 5–20 μg/ml is indicative of loss of cytoplasmic constituents from the cells. The increase in extinction of "protoplast" and cell suspensions which occurred in high drug concentrations is indicative of increased light scattering properties of the forms possibly caused by adsorbed chlorhexidine molecules (Hugo & Longworth, 1964).

EFFECT OF PRETREATMENT OF *B. megaterium* CELLS WITH CHLORHEXIDINE ON SUBSEQUENT TRANSFORMATION TO PROTOPLASTS

B. megaterium cells were suspended in water to contain 3·88 mg dry wt cells/ml and 5 ml of this suspension was added to 5 ml volumes of chlorhexidine solutions. After 30 min contact at 20° the cells were harvested by centrifugation washed in 10 ml of water and again harvested and resuspended in 10 ml water. 1 ml of this suspension was then added to 9 ml of a solution such that the final suspension contained 0·194 mg dry wt cells/ml, 20 μg/ml lysozyme 0·5M sucrose in 0·013M phosphate

buffer pH 7·3 at 20°, conditions previously determined as optimal for protoplast production. The control experiments consisted of cells treated with water before addition to lysozyme and cells examined after chlorhexidine treatment, omitting lysozyme treatment. After various time intervals in the lysozyme medium samples were examined by interference microscopy. The results are presented in Table 1.

TABLE 1. MICROSCOPIC APPEARANCE OF *B. megaterium* CELLS AFTER LYSOZYME TREATMENT (20 μg/ml) FOLLOWING 30 min CONTACT WITH CHLORHEXIDINE DIACETATE SOLUTIONS

Conc. of chlorhexidine diacetate pretreatment μg/ml	Period of contact with lysozyme		
	1 hr	3 hr	18 hr
0	conversion to spherical forms complete	spherical forms survive but some lysis evident	few spherical forms, much lysed matter
5	95% conversion to spherical forms	" "	" "
10	some spherical forms and lysed cells	much lysed matter	much lysed matter
20	rods and lysed cells	lysed matter and a few rods	lysed matter
50			
100			
200	rod forms only, no lysis	rod forms only, no lysis	rod forms only, no lysis
400			
500			

Cells treated with chlorhexidine but not exposed to lysozyme remained unchanged as rods. Lysozyme treatment of chlorhexidine-treated cells showed that in low concentrations the drug destroyed the permeability barrier which is responsible for maintaining lysozyme-produced protoplasts in hypertonic media. High concentrations of the drug rendered the cells insensitive to lysozyme action and inhibited lysis.

TABLE 2. EFFECT OF CHLORHEXIDINE DIACETATE AND CTAB ON THE GRAM STAINING REACTION OF *Staph. aureus*

Concn. of drug μg/ml	% of cells remaining Gram-positive after contact with drug, hr.							
	Chlorhexidine				CTAB			
	1	3	6	24	1	3	6	24
0	100	100	90	50	100	100	98	50
10	100	100	80	20	100	75	50	30
20	100	90	70	30				
30	100	90	60	10				
40	100	90	50	20				
50	100	90	50	5	100	95	50	40
100	100	90	50	5	100	95	70	50
200	100	90	50	5				
300	100	90	50	2				
500	100	90	50	2	100	100	100	98
800	100	90	40	2	100	100	100	98

PREPARATION OF SPHEROPLASTS OF *E. coli*

The method used was that of Hugo & Russell (1960): 10 ml of an 18 hr culture of *E. coli* in nutrient broth was added to 190 ml of the conversion medium containing 100 units/ml benzylpenicillin (sodium salt) and the

turbidity of the culture and the number of spheroplasts were determined hourly after incubation at 37°. The experiment was repeated but the cells were treated with 100 μg/ml chlorhexidine for 10 min before addition to the conversion medium. The results are presented in Fig. 2; no spheroplasts were produced when the cells were pretreated with chlorhexidine.

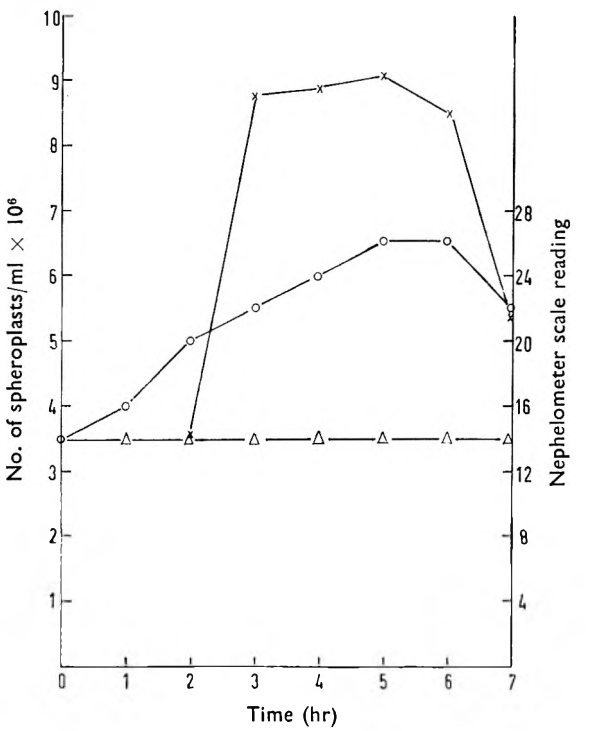

FIG. 2. Changes in turbidity (O—O) and number of spheroplasts (X—X) when culture of *E. coli* is treated with 100 units/ml benzylpenicillin in hypertonic medium. Also, changes in turbidity (△—△) of *E. coli* culture, pretreated with chlorhexidine diacetate 100 μg/ml for 10 min, in hypertonic medium containing 100 units/ml benzylpenicillin.

EFFECT OF CHLORHEXIDINE ON PENICILLIN-INDUCED SPHEROPLASTS OF *E. coli*

Spheroplasts were produced as described above and 4 hr after inoculation 9 ml samples were removed and added to 1 ml volumes of chlorhexidine in 0·33M sucrose at 20°, and the changes in extinction at 500 mμ followed. The results, presented in Fig. 3, show that chlorhexidine causes lysis of spheroplasts.

EFFECT OF CHLORHEXIDINE AND CTAB ON THE GRAM STAINING REACTION OF *Staph. aureus*

Dawson, Lominski & Stern (1953), found that low concentrations of CTAB caused loss of ability of *Staph. aureus* cells to retain the crystal violet–iodine complex of the Gram staining reaction whilst high

concentrations of the drug inhibited the conversion of cultures suspended in water to the gram-negative state.

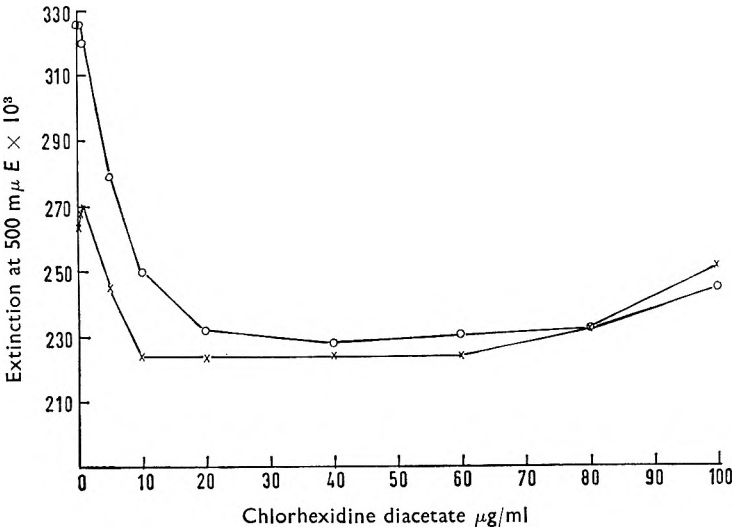

FIG. 3. Effect of chlorhexidine diacetate on penicillin-induced spheroplasts of *E. coli*. O—O Extinction ($E \times 10^3$) after 5 min contact. X—X Extinction ($E \times 10^3$) after 1 hr contact.

Cell suspensions containing *Staph. aureus* (1·2 mg dry wt cells/ml) were treated with various concentrations of chlorhexidine or CTAB at 20°, samples were Gram stained by the method of Preston (1962) and an estimate made of the percentage of Gram-positive cells. Table 2 shows that both compounds caused transformation to Gram-negative forms in low concentrations but the change was inhibited in high concentrations of CTAB.

Discussion

It is generally accepted that, underlying the rigid wall of a bacterial cell, there exists an osmotic barrier which is semipermeable and which bounds the contracting cytoplasm during plasmolysis (McQuillen, 1960b; Hughes, 1962). The membrane is also thought to be responsible for the control of diffusion into and out of the cell and is believed to be the site of action of many enzyme systems (Hughes, 1962).

Mitchell (1962) points out that to produce the spatial and metabolic organisation of the organism the enzymes must be supported upon or within a specifically locating framework and that the framework or backbone of the whole spatial metabolic organisation is in bacterial cells, the cell wall and cytoplasmic membrane. Hence any compound or circumstance which causes damage to this framework will upset the ordered reactions of the cell.

Our results show that chlorhexidine in low concentrations caused lysis

of osmotically sensitive forms suspended in hypertonic media and that higher concentrations affected the cell and rendered it insensitive to osmotic shock. Further it may be noted that treatment of cells with chlorhexidine destroyed their ability to undergo transformation to spherical forms on removal of, or damage to, the cell wall. Low concentrations of drug appeared to damage the cytoplasmic membrane of *B. megaterium* so that subsequent lysozyme treatment produced lysis and high concentrations fixed the cells in the rod shaped form. Possible mechanisms for this latter effect are: prevention of the action of lysozyme; denaturation or destruction of the cytoplasmic membrane; or physical sealing in of the cell surface caused by a build up of multilayers of drug around the cell.

If one assumes that only living bacteria possessing an intact cytoplasmic membrane and cytoplasm are able to undergo spherical transformation, then lack of this ability is indicative of death. The inability of chlorhexidine-treated cells to undergo spherical transformation in the presence of penicillin may be explained by the fact that spheroplasts are believed to be formed as a result of unbalanced cell wall synthesis, that is, where synthesis of the backbone mucopeptide of the cell wall is prevented by penicillin. If chlorhexidine reacts with the cells so as to destroy their viability, then no such transformation could occur.

Dawson, Lominski & Stern (1953) suggest that the inhibition of leakage of cell constituents caused by high concentrations of CTAB is a result of protein denaturation and inactivation of autolytic enzymes. It is accepted that damaged cells of *Staph. aureus* do not retain the crystal violet-iodine complex of the Gram stain and that damage to the cytoplasmic membrane and cell wall of this organism will cause a loss of Gram-positive staining (Salton, 1961). Dawson & others (1953) explain the retention of the crystal violet-iodine complex by cells treated with high concentrations of CTAB in terms of protein denaturation and cytoplasmic coagulation. However, no such effect is noted with chlorhexidine and high concentrations of this drug, which prevent lysis of osmotically sensitive forms and tend to inhibit leakage of cell constituents (Hugo & Longworth, 1964) are more effective in transforming cells to the Gram-negative state than are low concentrations which cause both lysis and leakage.

Autolytic enzymes cannot be involved in the observed lysis of "protoplasts" and spheroplasts, since where lysis occurred it was practically complete within 1 hr. It may be that inhibition of lysis and leakage by chlorhexidine is caused by a build up of multilayers of the drug on the cell surface; these layers are washed off by the iodine-acetone used in the decolourisation stage of the Gram staining procedure so that the reagent can then penetrate to decolourise the cell.

It would appear, therefore, that chlorhexidine exerts its bactericidal action by combination with the cell surface and disruption of the permeability barriers of the cell and that the subsequent manifestation of this damage in terms of the release of cell constituents plays only a minor role in the mode of action. The primary action of the drug appears to be

a disruption and disorganisation of the structure and function of the cytoplasmic membrane.

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Letters to the Editor

The inhibition of analgesia in mice by thiopentone

SIR,—Clutton-Brock (1960, 1961) and Dundee (1960) have independently demonstrated that sub-anaesthetic doses of thiopentone antagonize the analgesic action of pethidine and nitrous oxide in man. We are now reporting a similar investigation in mice using morphine, heroin, pethidine and codeine.

Groups of male mice (18–24 g) were injected subcutaneously with the appropriate analgesic and the ED50 determined. The experiment was then repeated on additional groups of mice which were injected intraveously with 25 mg/kg of sodium thiopentone in addition to the analgesic and the ED50 re-determined.

Analgesia was evaluated by the tail-clip technique of Bianchi & Franceschini (1954). The method of Miller & Tainter (1944) was used to determine the ED50's. The parameters obtained from the two experiments were treated statistically.

TABLE 1. THE EFFECT OF THIOPENTONE ON ANALGESIA

	Analgesic only E.D.50 ± s.e.	Analgesic plus thiopentone E.D.50 ± s.e.	P
	mg/kg	mg/kg	
Morphine	15 ± 2·4 (60)	43 ± 11·5 (25)	<0·05
Heroin	0·95 ± 0·2 (35)	3·6 ± 0·28 (25)	<0·05
Pethidine	28 ± 3·2 (35)	54 ± 4·7 (20)	<0·05
Codeine	62 ± 11·4 (25)	110 ± 16 (20)	<0·05

Figures in brackets indicate the number of mice used.

Table 1 shows that the ED50 for all the analgesics was significantly higher for the thiopentone-treated mice than for the controls.

These experimental findings and those of Neal (1964) are in agreement with the clinical observations that thiopentone antagonizes the analgesic action of pethidine.

From the work of Brodie & others (1950), Clutton-Brock (1960, 1961) has concluded that the anti-analgesic action in man is related to the plasma level of thiopentone and Dundee (1960) relates it to the presence of sub-anaesthetic doses of thiopentone in the brain. There is no satisfactory explanation for the increased sensitivity to pain after thiopentone, though it has been suggested that the reticular system of the brain stem may be involved.

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September 14, 1964

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An independent nerve-pathway for 5-hydroxytryptamine in the guinea-pig ileum

SIR,—5-Hydroxytryptamine, like dimethylphenylpiperazinium or nicotine, contracts the longitudinal muscle of the guinea-pig ileum mainly by an action through a cholinergic nerve-pathway (Day & Vane, 1963). The evidence obtained from the selective use of autonomic blocking agents (Brownlee & Johnson, 1963) showed that 5-hydroxytryptamine activates receptors on the intramural parasympathetic ganglion cells which are pharmacologically distinct from the receptors activated by dimethylphenylpiperazinium or nicotine: the end result of this ganglion stimulation is the release of acetylcholine from the parasympathetic post-ganglionic nerve-terminals.

The question has been raised (Brownlee & Johnson, 1963) whether the specific ganglion receptors for 5-hydroxytryptamine are located on a cholinergic nerve-pathway which is separate from that stimulated by nicotine or dimethylphenylpiperazinium. Evidence in favour of this possibility has been obtained from experiments made to demonstrate the effect on the responses to drugs of the time course of inhibition of the cholinesterases of the guinea-pig isolated ileum by *NN*-diisopropylphosphorodiamidic fluoride (mipafox).

Harry (1962) and Johnson (1963a,b) based their choice of concentration of mipafox on the evidence obtained by J. Harry and G. D. H. Leach in this department in March, 1960. The figures they obtained for the inhibition of the esterases of the homogenised ileum of the guinea-pig by mipafox are reproduced below with their permission in Table 1. The relation between the concentration of mipafox and the inhibition per cent of the esterases whose substrate was butyrylcholine is given in Fig. 1 along with the relation between the mipafox concentration and the inhibition of the esterases whose substrate was acetylcholine, from which it is seen that butyrylcholinesterase is completely inhibited by 1×10^{-6} Molar and acetylcholinesterase by 1×10^{-5} Molar mipafox. With a molecular weight of 182 for the inhibitor the concentration for acetylcholinesterase inhibition becomes $1{\cdot}8 \times 10^{-6}$ g/ml. This figure of 1·8 μg/ml for the inhibition of all esterases of the homogenised guinea-pig ileum which hydrolysed acetylcholine provided the basis for the working concentration of 10 μg/ml of mipafox used in these experiments.

TABLE 1. INHIBITION OF ESTERASES OF HOMOGENISED ILEUM OF THE GUINEA-PIG BY MIPAFOX AT 37°C.

Manometer	Final molar conc.	−Log molar conc.	% Residual activity	% Inhibition
A	Control		100	0·0
B	$7{\cdot}5 \times 10^{-6}$	5·13	2·8	97·2
C	$7{\cdot}5 \times 10^{-7}$	6·13	31·96	63·04
D	$1{\cdot}5 \times 10^{-7}$	6·82	33·13	66·87
E	$7{\cdot}5 \times 10^{-8}$	7·13	44·72	55·28
F	$7{\cdot}5 \times 10^{-9}$	8·13	95·82	4·18
	Substrate Acetylcholine 0·0138M Total evolution of CO_2 from control manometer = 7,737 µl. CO_2/g/hr			
G	Control		100	
H	$7{\cdot}5 \times 10^{-6}$	5·13	2·01	97·99
J	$7{\cdot}5 \times 10^{-7}$	6·13	1·96	98·04
K	$7{\cdot}5 \times 10^{-8}$	7·13	23·15	76·85
L	$7{\cdot}5 \times 10^{-9}$	8·13	100·0	0·0
M	$7{\cdot}5 \times 10^{-10}$	9·13	100·0	0·0
	Substrate Butyrylcholine 0·0138M Total evolution of CO_2 from control manometer = 18,217 µl. CO_2/g/hr			

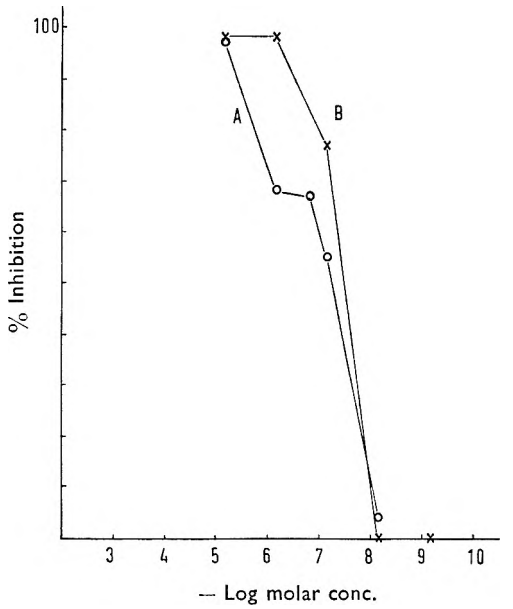

FIG. 1. The relation between the concentration of mipafox and the inhibition % of the acetylcholinesterase (A) and the butyrylcholinesterase (B) of the homogenised guinea-pig ileum (Warburg technique). Acetylcholine and butyrylcholine were each 0·0138 molar. Butyrylcholinesterase was completely inhibited by 1×10^{-6} molar (0·18 μg/ml) and acetylcholinesterase by 1×10^{-5} molar (1·8 μg/ml) mipafox (Harry, J. & Leach, G. D. H., unpublished results, 1960).

Treatment of the isolated ileum for 60 or 75 min with mipafox (10 μg/ml) potentiated maximally the responses to acetylcholine, 5-hydroxytryptamine and dimethylphenylpiperazinium but left the responses to histamine unaffected (Fig. 2). Further, the responses to 5-hydroxytryptamine were potentiated to a statistically significantly greater extent than either acetylcholine or dimethylphenylpiperazinium which, within the limits of the experimental error, were both potentiated to the same extent. The potentiation (measured as a displacement of the dose-response curve at a level of the E.D. 50 response) of the responses to 5-hydroxytryptamine was 0·927 $\pm$ 0·153 (log units $\pm$ s.e.), to dimethylphenylpiperazinium 0·486 $\pm$ 0·132 and to acetylcholine 0·375 $\pm$ 0·054. These potentiations in log units correspond to about eight-fold for 5-hydroxytryptamine, three-fold for dimethylphenylpiperazinium and two-fold for acetylcholine. Robertson (1954) also obtained similar results on the rabbit ileum treated with the acetylcholinesterase inhibitor 1,5-di(*p*-allyl-*N*-methylaminophenyl)-pentan-3-one dimethobromide.

After treatment with mipafox for only 1·5 min the responses to acetylcholine and dimethylphenylpiperazinium were potentiated, and this potentiation remained steady until after treatment for 40 min when the responses to these drugs were potentiated further. The 5-hydroxytryptamine responses were not potentiated by mipafox until after the 40 min treatment period when they were maximally potentiated. The responses to histamine were not significantly different from the control responses even after 80 min treatment with mipafox.

The differences in the degree of potentiation of 5-hydroxytryptamine on the one hand and dimethylphenylpiperazinium and acetylcholine on the other,

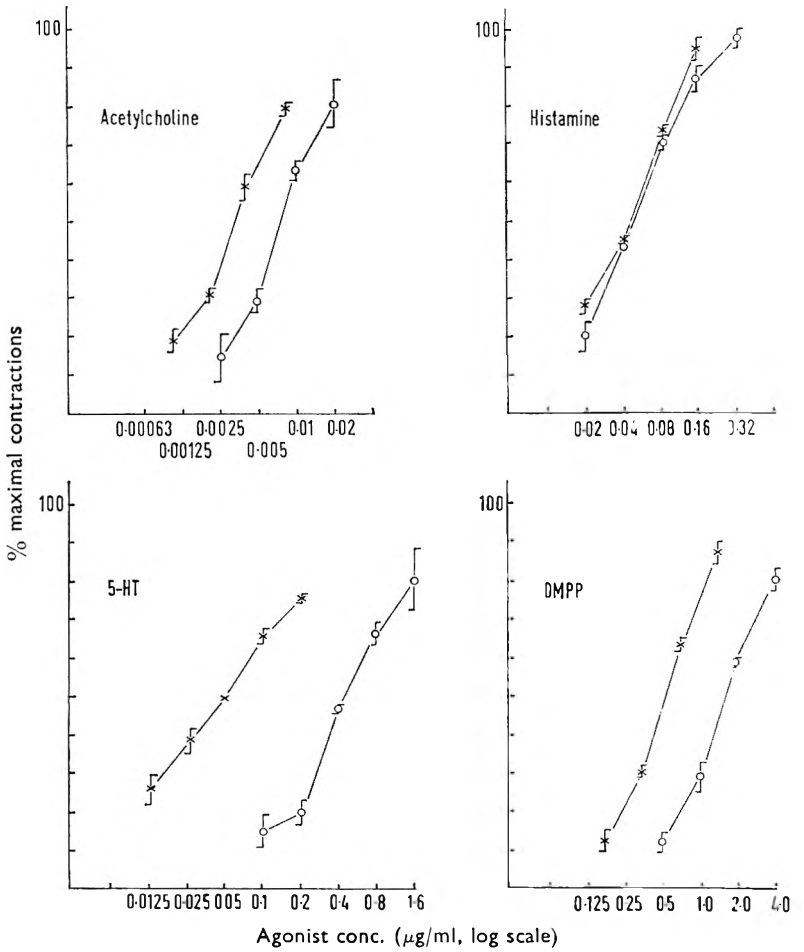

FIG. 2. The effect of complete inhibition of the cholinesterases of the guinea-pig ileum by treatment with mipafox (10 μg/ml) for 1 hr on its responses to acetylcholine, histamine, 5-hydroxytryptamine and dimethylphenylpiperazinium. ○ Responses to the agonists. × Responses after treatment with mipafox for 1 hr (s.e. is also shown). The curves for acetylcholine, 5-hydroxytryptamine and dimethylphenylpiperazinium were displaced to the left so that they were parallel to the originals. The histamine responses were unchanged. 5-Hydroxytryptamine was potentiated to a statistically significantly greater extent than either acetylcholine or dimethylphenylpiperazinium.

and the differences in the time course of inhibition of the cholinesterases on the responses of the ileum to drugs, are unexpected if dimethylphenylpiperazinium and 5-hydroxytryptamine are activating the same cholinergic nervous system, and furthermore these differences cannot be explained by the two drugs releasing different amounts of acetylcholine from the same nerve-endings since recent work in this department (Johnson, 1963, communicated to the British Pharmacological Society at Dublin) showed that equi-effective doses of the two drugs released equal amounts of acetylcholine; yet it was this acetylcholine which mipafox potentiated to a different degree when released by 5-hydroxytryptamine

from that released by dimethylphenylpiperazinium. In addition, the different time course of inhibition implied that the acetylcholine was liberated under different circumstances by the two drugs.

The known property of mipafox to discriminate between the two types of cholinesterase (Fig. 1.; Holmstedt, 1957) would favour the explanation that the acetylcholine released by 5-hydroxytryptamine is hydrolysed by a cholinesterase with different properties from that which hydrolyses the acetylcholine released by dimethylphenylpiperazinium or acetylcholine added exogenously, and it is difficult to imagine how this could arise unless 5-hydroxytryptamine is acting on a nerve-pathway independent from that activated by dimethylphenylpiperazinium. The greater potentiation of 5-hydroxytryptamine than dimethylpiperazinium by mipafox can be explained only by a difference in the amount of or nature of the cholinesterase at a separate nerve-ending.

The simplest explanation suggested by these experiments is that 5-hydroxytryptamine and dimethylphenylpiperazinium activate different nerve-pathways; the acetylcholine released by 5-hydroxytryptamine being hydrolysed mostly by acetylcholinesterase whereas that released by dimethylphenylpiperazinium, and also exogenous acetylcholine, being hydrolysed by a mixture of both butyryl and acetylcholinesterase.

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Structural consideration in the inhibition of rat brain acetylcholinesterase

SIR,—The presence of a negatively charged "anionic" site responsible for the binding of substituted ammonium ion, and an 'esteratic' site which binds the ester group is well established for acetylcholinesterase. Many quaternary ammonium compounds including neostigmine have been shown to be powerful inhibitors of this enzyme (Augustinsson & Nachmansohn, 1949). The preferential inhibition of true acetylcholinesterase by neostigmine without equally effecting other esterases (Wilson, Levine & Freiberger, 1952) and the presence of mainly true acetylcholinesterase in rat brain (Parmar, Sutter & Nickerson, 1961) led us to investigate the effect of tetramethylene, hexamethylene, octamethylene, decamethylene and dodecamethylene-bis(3-dimethylaminophenyl *N*-methylcarbamate) dimethobromides on acetylcholinesterase activity of rat brain homogenate, in order to show the role of the number of methylene groups connecting two neostigmine molecules present in these compounds.

Acetylcholinesterase activity in brain homogenate was estimated colorimetrically (McOsker & Daniel, 1959) using acetylthiocholine as the substrate

where hydrolysis resulted in thiocholine which was estimated by the nitroprusside reaction. Acetylcholinesterase activity of various subcellular fractions of rat brain determined colorimetrically with acetylthiocholine has been shown to be almost identical with the activity determined manometrically using acetylcholine or acetylthiocholine as the substrate (Parmar, Sutter & Nickerson, 1961). Inhibitory effects of neostigmine derivatives, at final concentration of 3×10^{-8}M, on acetylcholinesterase during hydrolysis of acetylthiocholine is shown in Table 1.

TABLE 1. INHIBITION OF ACETYLCHOLINESTERASE IN RAT BRAIN HOMOGENATE

Preincubation time Min	% inhibition					
	Neostigmine	$[CH_2]_4$*	$[CH_2]_6$*	$[CH_2]_8$*	$[CH_2]_{10}$*	$[CH_2]_{12}$*
0	0·0	0·0	3·9	7·7	16·0	27·0
	31·6	16·3	44·0	56·0	70·8	75·0
10	32·6 (31·9)	16·3 (15·9)	44·0 (44·3)	54·2 (55·9)	68·8 (69·0)	75·0 (74·2)
	31·6	15·0	45·0	57·5	67·5	72·5
20	61·0	65·4	71·6	79·3	92·0	100·0
30	71·6	73·1	73·5	80·7	100·0	100·0

* Bis (3-dimethylaminophenyl *N*-methylcarbamate) dimethobromide.
Enzyme activity was determined as change in extinction per 100 mg wet tissue during 10 min incubation. Each tissue sample was done in triplicate. The reaction mixture in a volume of 2 ml contained tris buffer (43·7 mM) pH 7·4, sodium chloride (350 mM), acetylthiochline ($1·5 \times 10^{-3}$M), 0·2 ml of 10% brain homogenate and inhibitors at the final concentration of 3×10^{-8}M. Suitable controls for substrate and tissue blanks were taken. The preincubation time denotes incubation of brain homogenate with the inhibitor for 10, 20 and 30 min before adding acetylthiocholine. Experiments with 0 time preincubation denote when both substrate and inhibitor were added at the same time. Figures in parentheses are averages.

Increase in the number of methylene groups in the compounds was found to increase enzyme inhibition. Inhibition also increased on preincubation of the enzyme preparation with these compounds for varying length of time before the addition of acetylthiocholine. Inhibitory effects of these derivatives could thus be modified by the substrate, indicating presumably the competitive nature of the inhibition. Our results are in good agreement with those reported earlier (Kraupp, Stumpf, Herzfeld & Pillat, 1955) where inhibition of dog serum and erythrocyte cholinesterase similarly increased with the length of the polymethylene chain of these compounds.

Our results indicate that true acetylcholinesterase possesses two 'anionic' sites to bind both quaternary ammonium groups of such bis-quaternary ammonium compounds (Bergmann, Wilson & Nachmansohn, 1950). The presence of the polymethylene chain in these derivatives may be assumed to facilitate such binding. Increase in the number of such methylene groups makes the compound more flexible which ultimately results in greater ease in binding to two "anionic" sites and thereby producing increased inhibition. Further studies may elucidate the exact role of such a polymethylene chain in the inhibition of brain acetylcholinesterase.

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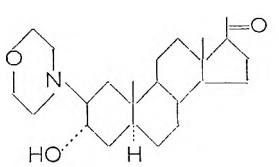
Anticonvulsant and interneuronal blocking activity in some synthetic amino-steroids

SIR,—The electroshock seizure threshold in animals can be raised or lowered by adrenocortical and sex hormones (Woolley & Timiras, 1962a,b and ref. cit.; Woodbury 1958 and ref. cit.). So far only limited success has been achieved in the search for synthetic steroids with potent central nervous system activity, yet devoid of hormonal actions, e.g. Brown & Sarett (1963). Certain amino-esters of 21-hydroxypregnanedione possess general anaesthetic activity (Figdor & others, 1957) and funtumidine (3α-amino-20α-hydroxy-5α-pregnane) is reported to cause tranquillisation (Blanpin & Quevauviller, 1960, and references cited).

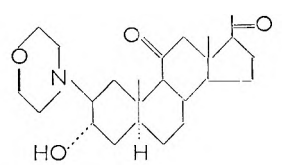
We have investigated a series of amino-steroids for ability to produce loss of righting reflex. In addition, anti-tremorine activity, antagonism to electrically- and chemically-induced seizures and effects on blood pressure, neuromuscular transmission and the crossed extensor reflex in the cat have been examined.

The compounds tested were derivatives of androstane or pregnane in which the amino-radical (amino-, n-propylamino-, n-butylamino-, dimethyl-amino-, diethylamino-, piperidino- and morpholino-) was attached to C-2, C-6 or C-16.

Of the four related compounds, 3α-hydroxy-2β-morpholino-5α-pregnan-20-one (I), 3α-hydroxy-2β-morpholino-5α-pregnane-11, 20-dione (II), 3α-hydroxy-16α-methyl-2β-morpholino-5α-pregnan-20-one (III) and 3α-hydroxy-16α-methyl-2β-morpholino-5α-pregnane-11, 20-dione (IV), the least substituted (I), was the most potent in causing loss of righting reflex.



I



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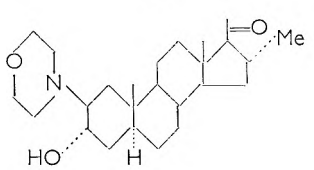
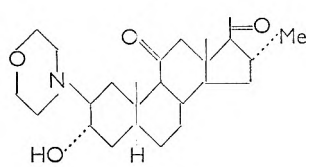


III



IV

Introduction of an 11-keto-group (II) into 3α-hydroxy-2β-morpholino-5α-pregnan-20-one (I) shortens the duration and only slightly increases the degree of this activity; introduction of a 16α-methyl group (III) into (I) lengthens the duration but much reduces the degree of activity. Compound IV, which has both an 11-keto-group and a 16α-methyl group, shows the influence of both substituents; the 16α-methyl group reduces the degree of activity of (II) but increases its duration; the 11-keto group slightly increases the degree of activity of (III) but reduces its duration. The net result relative to (I) is both a diminution of activity (16α-methyl group) and a shortening of the loss of the righting reflex (the effect of the 11-keto group being greater than that of the 16α-methyl group).

Compound V, 3α-hydroxy-2β-morpholino-5α-androstan-17-one, has ED50 and LD50 values for loss of righting reflex much greater than those for (I), although the ED50/LD50 ratio for (V) was the lower.

Compound (VI), 3β,17α-dihydroxy-16β-morpholino-5α-pregnane-11, 20-dione, was the only member of a 16-amino series tested to cause loss of the righting reflex at a dose well below the LD50; both the ED50 and LD50 values were, however, much higher than the corresponding values for the 2β-analogues (Table 1).

TABLE 1. ED50 (LOSS OF RIGHTING REFLEX), DURATION OF EFFECT, LD50 AND ED50/LD50 RATIO IN MICE

Compound number	ED50 (mice) mg/kg (±s.e.)	Dose* mg/kg	Duration of loss of righting reflex in min (±s.e.)	LD50 mg/kg (±s.e.)	Ratio LD50/ED50
I	13 ± 1·63	26	30 ± 5·9	61 ± 2·65	4·69
II	11·8 ± 1·13	24	16·9 ± 1·8	56 ± 1·89	4·75
III	29 ± 0·67	58	37·7 ± 4·8	82 ± 1·42	2·83
IV	19·2 ± 0·84	40	23 ± 1·61	71 ± 2·2	3·64
V	180 ± 6·5			340 ± 7·1	1·89
VI	45·5 ± 1·6			107 ± 3·1	2·38
Mephenesin	99 ± 5·7			174 ± 8·2	1·79

* Twice ED50.

Loss of righting reflex is induced by general anaesthetics and neuromuscular and interneuronal blocking agents (O'Dell, 1960). As our compounds did not produce general anaesthesia and had no observed effect on the gastrocnemius muscle–sciatic nerve preparation of the anaesthetised cat, loss of righting reflex was not due to general anaesthesia or neuromuscular blockade. Compounds (I–IV) reduced the crossed extensor reflex in the spinal cat, suggesting that the observed effect was due to interneuronal blockade; a view which is favoured by the observation that, in mice, the pinna reflex disappeared before the corneal reflex (Witkin & others, 1959). The ED50/LD50 ratios of compounds I–VI are higher than that of mephenesin (Table 1).

No anti-electroshock activity was noted after intravenous injection of any compound without simultaneous sedation or a partial loss of righting reflex or both of these.

Compound (I) was the most interesting member of the series in that the intravenous dose required to antagonise the convulsant action of 120 mg/kg of leptazol given intraperitoneally (Berger, 1954) was 3·6 mg/kg, which was one-seventeenth of the LD50 and approximately one-quarter of the ED50 value for loss of the righting reflex. Increasing the time between the injection of (I) and of leptazol from 5 to 20 min, led to a twofold increase in the PD50 (protective) value (6·3 mg/kg) which, nevertheless, was still lower than the ED50 value for loss of righting reflex. A search of the literature has so far revealed no other synthetic steroid possessing marked anti-leptazol activity.

None of the six above-mentioned compounds, on intravenous administration, antagonised tremorine-induced tremors.

For optimum activity in producing loss of righting reflex and anti-leptazol activity, the following requirements appear to be the necessary minimum—(*a*) a pregnane nucleus, (*b*) a 2β-morpholino-group, (*c*) a 3α-hydroxyl group.

Although the amino-esters of 21-hydroxypregnanedione of Figdor & his colleagues (1957) had general anaesthetic activity and the compounds (I–VI) we have investigated possessed loss-of-righting-reflex and anti-leptazol activities, there is agreement on two points: (*a*) the morpholino-substituted steroid was the most active and (*b*) nuclear substitution decreased potency.

A further compound, (VII), 3β-acetoxy-5α-hydroxy-6β-morpholino-5α-pregnan-20-one, produced convulsions in mice and these superficially resembled leptazol convulsions.

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A sensitive preparation for the assay of 5-hydroxytryptamine

SIR,—The crop of the young chick contracts strongly in response to minute amounts of 5-hydroxytryptamine (5-HT) and has been used as a sensitive assay preparation.

Chicks (Silver Link) up to 7 days old were starved overnight to empty the crop and then killed with ether. The crop was removed and placed in a petri-dish containing Krebs-Henseleit solution (g/litre: NaCl 6·95, KCl 0·34, $CaCl_2$ 0·28, KH_2PO_4 0·162, $MgSO_4$ 0·294, $NaHCO_3$ 2·1 and dextrose 2) at room temperature. It was opened by a longitudinal cut in the wall of the attached portion of oesophagus and a strip of tissue about 3 mm wide was then cut transversely from the middle of the opened crop. The strip was suspended in a Perspex bath of just over 1 ml capacity containing Krebs solution at room temperature (20-23°) which was bubbled slowly with 5% carbon dioxide in oxygen. One end of the strip of crop was attached to an isotonic frontal writing lever loaded with 4 g and magnifying the contractions 12 times. An interval of 30-60 min was

allowed before beginning the assay; during this time the muscle relaxed maximally. Strips cut from crops which had contained food when the chicks were killed, usually exhibited powerful and erratic spontaneous movements and were unsuitable for assay preparations. Some strips of crop taken from starved chicks also exhibited spontaneous movements but these were rarely troublesome since they were regular and small in amplitude.

The standard solutions and the solution to be analysed were first diluted with Krebs solution so that the required doses were contained in volumes of 1 ml. Doses were added by emptying the bath from the bottom and refilling from the top with 1 ml of the required solution delivered from a pipette. The drug was left in contact with the tissue for 60 sec and then washed out by refilling the bath twice with fresh Krebs solution. Doses were added in the form of a Latin square design at constant intervals of 6 to 9 min. The preparations remained stable throughout at least 2 complete Latin squares (32 doses) and many were sensitive to as little as 10^{-12} g (1 picogram) of 5-HT. At room temperature the weak solutions of 5-HT remained stable throughout the assay. Raising the temperature of the Krebs solution to 32° slightly increased the sensitivity of the assay. However, it was then necessary to maintain the 5-HT solutions at the same temperature and this caused progressive decomposition. In 7 assays, the results of which were analysed statistically, the indices of precision (λ) were less than 0·05 and the fiducial limits of the potency ratios all fell between 88 and 112%.

The preparation was also found to be sensitive to histamine (about 1 ng/ml) and acetylcholine (about 10 ng/ml in the absence of anticholinesterase) and preliminary experiments suggest that it may be used to assay these substances also. Each agonist was selectively blocked by an appropriate antagonist so that any one might be assayed in the presence of the other two. Histamine was completely blocked by mepyramine (10 ng/ml), 5-HT by bromolysergic acid diethylamide (1-5 μg/ml) and acetylcholine by atropine (10 ng/ml). This concentration of atropine approximately halved the sensitivity to 5-HT but even in the presence of atropine the preparation was considerably more sensitive than other available preparations. Because of the high sensitivity, body fluids to be assayed must be extensively diluted with Krebs solution and it is hoped that interfering substances may thereby be inactivated. Assays made on samples obtained from 6 normal adults indicate that the method is suitable for the estimation of free 5-HT in urine.

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Effects of ammonium chloride and sodium bicarbonate on resistine levels in rats.

SIR,—Different tissue-damaging procedures result in enhanced production of "resistine" (Karady & Kovacs, 1948), a substance which exerts an antihistamine action and which reduces histamine release (Kardy, Gecse & Horpacsy, 1962). Since acidifying or alkalizing treatments often produce favourable results in clinical practice (mainly in allergic disease), it was of interest, in order to shed some light on the mechanism of the favourable effect of these treatments, to follow the changes in resistine levels in rats after treatment with acidifying and

alkalizing compounds. Resistine levels were measured by determining the ability of rat blood to protect guinea-pigs from convulsions produced by histamine (Prokai, Mustardy & Karady, 1961; Karady, Prokai & Mustardy, 1961). At the same time the ability of the blood to protect guinea-pig convulsions produced by 5-hydroxytryptamine (5-HT) and acetylcholine aerosols was tested to determine the specificity of resistine.

Groups of 14 or more rats received either ammonium chloride (680 mg/kg) or sodium bicarbonate (600 mg/kg) by stomach tube daily for 9 days. Twenty-four hr after the last dose, they were killed and the sera of rats belonging to the same group were mixed and injected intraperitoneally into groups of guinea-pigs which had been previously tested for sensitivity to aerosols of histamine (0·15%), 5-HT (0·2%) and acetylcholine (0·6%). Six hr later, the sensitivity to the three agents was re-tested. In each case, the time to the first convulsive cough was taken as the end-point, and the results are calculated as mean percentages of

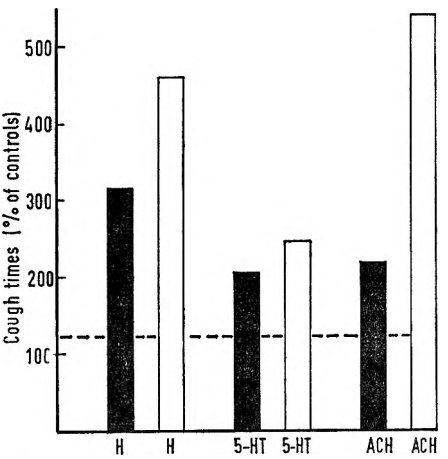

FIG. 1. Effect of sera of rats treated with ammonium chloride (■) or sodium bicarbonate (□) on the cough times of guinea-pigs subjected to histamine, 5-HT and acetylcholine aerosols. Values greater than the broken line are highly significant ($P<0.01$). Note that both treatments prolong the cough times in each case.

cough-times of guinea-pigs before treatment. The results recorded in Fig. 1 show that both treatments stimulated rats to produce resistine, as indicated by the significant increases in the histamine aerosol times. However, the effect is not specific as cough times to 5-HT and acetylcholine were also increased. This suggests that resistine is antihistamine, anti-5-HT and atropine-like in nature.

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Interaction between 5-hydroxytryptamine and antitumoural drugs

SIR,—The recent observation made by Uroic, Rabadjija & Supek (1964) about the increased toxicity of mustine hydrochloride (nitrogen mustard) in animals pretreated with 5-hydroxytryptamine (5-HT), prompted us to report data dealing with the interaction between 5-HT and alkylating agents.

Tumour-bearing female mice of Swiss strain, average weight 22 ± 2 g, kept in groups of 10 in Makrolon cages at room temperature (22°) and relative humidity 60%, were used in all experiments. Sarcoma 180 was implanted subcutaneously in the intrascapular area in fragments of 10 mg average weight. Ehrlich carcinoma was transplanted in the same area using 0·2 ml (about 3 million cells) of the ascite form.

Treatment started 6 days after transplantation of the tumour and was given daily for 6 days. Animals were killed two days after 5-hydroxytryptamine creatinine sulphate was given intraperitoneally, 10 min before DL-sarcolysine or 5-fluorouracil. The results obtained are reported in Table 1.

TABLE 1. COMBINATION OF 5-HT WITH DL-SARCOLYSINE OR 5-FLUOROURACIL IN TUMOUR-BEARING MICE**

Treatment (mg/kg/i.p. × 6 days)		Tumour	Tumour weight	Leucocyte counts	Spleen weight	Intestinal DNA
Saline		E	100 ± 8	100 ± 12	100 ± 9	100 ± 5
5-HT	62·5	"	110 ± 15	82 ± 12	68 ± 6	—
5-HT	250	"	75 ± 14	84 ± 13	61 ± 2	119 ± 6
DL-Sarcolysine	2·1	"	91 ± 15	83 ± 7	58 ± 5	—
5-HT +	62·5					
DL-Sarcolysine	2·1	"	79 ± 15	70 ± 8	38 ± 3	—
5-HT +	250					
DL-Sarcolysine	2·1	"	71 ± 12	43 ± 1	28 ± 3	—
DL-Sarcolysine	6·3	"	79 ± 11	37 ± 4	17 ± 1	90 ± 3
5-HT +	62·5					
DL-Sarcolysine	6·3	"	63 ± 13	54 ± 5	10 ± 2	—
5-HT +	250					
DL-Sarcolysine	6·3 *	"	31 ± 6	42 ± 5	9 ± 1	77 ± 4
5-Fluorouracil	29	"	51 ± 6	51 ± 4	51 ± 3	121 ± 16
5-HT +	250					
5-Fluorouracil	29	"	25 ± 3	27 ± 2	34 ± 3	79 ± 3
Saline		S180	100 ± 24	100 ± 15	100 ± 14	100 ± 3
DL-Sarcolysine	4·2	"	67 ± 19	19 ± 2	30 ± 3	94 ± 2
5-HT +	500					
DL-Sarcolysine	4·2	"	15 ± 4	7 ± 1	13 ± 1	90 ± 3
DL-Sarcolysine	6·3	"	48 ± 12	16 ± 1	14 ± 1	90 ± 5
5-HT +	500					
DL-Sarcolysine	6·3 †	"	21 ± 5	8 ± 1	10 ± 1	72 ± 5

* Mortality 3/8.
† Mortality 4/10.
E = Ehrlich carcinoma; S 180 = sarcoma 180.
** The effect of the various treatments are expressed as % of the control values (± s.e.). Intestinal DNA was determined according to Ceriotti (1955).

It is evident that 5-HT tends to increase the antitumoural effect of DL-sarcolysine particularly at given doses. However, the toxic effects of DL-sarcolysine on the leucocyte number, spleen weight and intestinal desoxyribonucleic acid (DNA) are also enhanced by 5-HT. Furthermore its effect is not specific for a nitrogen mustard derivative because the activity of 5-fluorouracil was also increased by the amine.

In other experiments, 5-HT at a dose of 250 mg/kg i.p., was given in Ehrlich carcinoma-bearing mice 10 min before equitoxic doses (1/2 of the LD50 determined after 6 days of treatment) of the following antitumoural drugs: DL-sarcolysine (6·3 mg/kg) cyclophosphamide (125 mg/kg) triethylenemelamine (1 mg/kg) 5-fluorouracil (29 mg/kg) and Mitomycin C (7-amino-9α-methoxy-mitosane) (2·65 mg/kg). On the 7th day the combinations 5-HT and triethylenemelamine and 5-HT and Mitomycin C were lethal for all the mice (10 per

group) whilst in the other combinations or treatments with the drugs alone no toxicity was recorded.

In our opinion the latter data show that the observed interactions between 5-HT and a nitrogen mustard derivative are not specific. Furthermore the effect of 5-HT is unlikely to have a therapeutical interest after systemic administration because of the large dose of 5-HT involved and the lack of a specific effect on the tumour growth.

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Book Review

THE QUANTITATIVE ANALYSIS OF DRUGS. By D. C. Garratt. Third edition. Pp. xiii + 925 (including Index). Chapman and Hall, London, 1964. 126s. U.K. only.

The appearance of the third edition of Dr. Garratt's well known book is timely. Concern that the standard of quality control of drugs should be maintained and improved has recently been shown both publicly and officially. Dr. Garratt is himself Chairman of a Pharmaceutical Society committee, the Science Committee (Pharmaceutical Analysis), whose efforts to improve both public awareness of and facilities for quality control of drugs are already showing considerable results.

The first edition of this book was published in 1937 and since that time it has been a source of knowledge for both the man on the bench and those who interpret his results. Reference to Garratt's book may not always tell you all the answers but it tells you a lot of them and is always a great help to morale in the difficult situations in which the pharmaceutical analyst sometimes finds himself.

The revised and enlarged edition now produced is correlated with the British Pharmacopoeia of 1958 and the United States Pharmacopeia XVI Edition. The book is in the main a series of monographs arranged alphabetically and covering 700 pages. The titles of these run from acetic acid to zinc, being in some cases the name of a particular drug and in others the name of a group. Examples are: colchicum, copper, cresol, halogen acids and salts, miscellaneous metallic compounds, phytomenadione, sulphurous acid. This arrangement is of value to those who become familiar with the layout of the book. For those who are not there is a good index. These general monographs are followed by a section on methods for determining synthetic organic substances not included in the general monographs. This is followed by sections on essential oils, and oils fats and waxes. The last 100 or so pages of the book are in the form of appendices on specific analytical techniques. These include: determination of alcohol, non-aqueous titration, tests for sterility, destruction of organic matter, etc. An appendix on interpretation of analytical results contains sections on slope ratio assays and sequential analysis.

Dr. Garratt and the team who have assisted him in the writing of this book are persons who have played a considerable part in the production and development of many of the methods described in it. As Dr. Garratt is himself, besides being President of the Society for Analytical Chemistry, the Chairman of its analytical methods committee, it is not surprising to find that many of the methods recommended in the book are those which have been studied and approved of by the S.A.C. The ultimate source of these and many other of the methods given affords ample assurance that they are the result of rigorous selection on severely practical grounds. References are given at the end of each monograph and appendix of the various sources used.

The book is well produced and strongly bound. It should be so, as it can be confidently predicted that it will be much used in close association with practical analysis on the bench.

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Letters to the Editor

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