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A phase-rule study of multiple-phase formation in a model emulsion system containing water, n-octanol, n-dodecane and a non-ionic surface-active agent at 10 and 25°

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A four-component emulsion system containing the non-ionic surfactant $C_8H_{17}(O{\cdot}CH_2{\cdot}CH_2)_6OH$ has been investigated, particularly in regions in which liquid crystal or more than two condensed phases are formed. Liquid crystal does not exist in the binary surfactant–water system at 25° but extensive regions form when the organic liquids octanol and dodecane are present, although these are not joined to the main two-liquid regions of the emulsion system. The surfactant is almost completely dissolved in the aqueous phase in water-dodecane mixtures but when substantial amounts of octanol are also present the partition coefficient is reversed in favour of the organic phase. At certain intermediate compositions three liquid phases are present. The significance of these results to the system as an emulsion are briefly discussed.

Although emulsions are treated by many workers as two-phase systems, there is increasing realization that more than two phases are frequently present, particularly in dispersions containing synthetic surfactants. The solubility of many surfactants in water is low, and in some instances limited not by the separation of a solid phase, but by liquid crystal (McBain, 1950). The situation is more complex in systems containing three components, which correspond to the simplest emulsions, and the phases present depend markedly on the nature of the third component, as well as on the properties of the surfactant (Lawrence, 1958; Mulley & Metcalfe, 1964; Mandell & Ekwall, 1968). It seems likely that some of the most important features of emulsions, for example emulsion-type, stability and viscosity, may in certain instances be explained by phase-rule studies (Salisbury, Leuallen & Chavkin, 1954; James & Goldemberg, 1960; Mulley, 1961; Burt, 1965; Lachampt & Vila, 1967; Friberg, Mandell & Larsson, 1969). A number of other workers in the emulsion field have referred to the multiphase character of their systems (Barry & Shotton, 1967; Talman, Davies & Rowan, 1967; Talman & Rowan, 1970). In practice, many emulsions have more than three components, although these are often not part of the emulsifying system; but a basic formula may commonly be considered to consist of four components, two being immiscible liquids and the others the emulsifying agent and the stabilizer. The properties of the system are often adjusted by varying the relative proportions of the two latter components.

The present paper describes a phase-rule study of such a model four component emulsion system containing an homogeneous non-ionic surfactant, a fatty alcohol as stabilizer, and a saturated hydrocarbon as the oil phase, with the object of finding

those regions of the system which form more than two condensed phases, as a preliminary to a study of the relation between the nature of the phases present and the properties of the system as an emulsion. A preliminary report describing a region where three liquid phases were found in this system, resulting in the formation of multiple-drop emulsions, was recently published (Mulley & Marland, 1970).

MATERIALS AND METHODS

Materials

n-Octanol (M.A. grade, Mann Research Laboratories Inc.) and an 'oil' phase of *n*-dodecane (BDH Ltd., 99% plus by g.l.c.) were used. The surfactant was 3,6,9,-12,15,18-hexaoxahexacosan-1-ol, [$C_8H_{17}.(OCH_2.CH_2)_6OH$] or C_8E_6 (Mulley, 1967). A pure sample was obtained by the following method. Sodium (5·3 g) was dissolved in triethylene glycol (137 g) and then heated with C_8E_3 Cl [prepared by treating C_8E_3 with a chlorinating mixture of thionyl chloride and pyridine (Mulley, 1958); 64 g, b.p. 109 to 110°/0·1 mm, m.p. −23·1°, n^{20} 1·4466]. Water (900 ml) was added and the product extracted with light petroleum (b.p. 100 to 120°, 600 ml, and 2 × 50 ml), at 70°. The organic phase was dried ($MgSO_4$) and fractionally distilled to give C_8E_6 (26%, b.p. 184 to 186°/0·1 mm, m.p. 7·5°, n_D^{20} 1·4528). Gingras & Bayley (1958) prepared C_8E_6 by a one-stage Williamson ether synthesis, while Corkill, Goodman & Ottewill (1961) used a two-stage process. The physical data recorded by these workers for their samples are (b.p. 180°/0·07 mm; n_D^{20} 1·4499 and b.p. 205°/0·0001 mm, f.p. 9·0, n_D^{40} 1·4463) respectively. The infrared spectrum measured with a Unicam S.P. 200 spectrophotometer confirmed the structure, but revealed traces of carbonyl and unsaturated impurity (Corkill & others, 1961). The compound was also assessed by g.l.c. (Mulley & Winfield, 1970). The results showed the compound to have a purity of at least 98 to 99%.

Phase-rule measurements

The binary, ternary and quaternary systems were investigated by a combination of synthetic (Mader, Vold & Vold, 1959), and analytical methods at 25°. The C_8E_6–water binary system was studied between 2 and 90°, and the ternary and quaternary systems at 10°, though less comprehensively than at 25°, and only by the synthetic method.

(a) *Synthetic method*

The quaternary system was systematically investigated by preparing ternary mixtures of composition suitable for studying the various phase boundaries, but all lying in one of several triangular sections through the tetrahedral model. The triangular planes begin as lines in the C_8E_6–water–dodecane face, which run from the C_8E_6 apex, and cut the water–dodecane axis at fixed ratios. Addition of octanol to a three-component mixture on one of the lines in the C_8E_6–water–dodecane face, alters the composition along a straight line drawn from the initial composition to the octanol apex, and maintain the ratios of the first three components.

The mixtures were prepared in glass ampoules sealed with rubber teats; additions to the ampoules were made using a micro-syringe. They were equilibrated for periods ranging from 5 min to 7 days before visual, and in some instances microscopical examination. Problems of equilibration were found, particularly in regions where

two-liquid plus liquid crystal regions were thought to exist. Gel-like regions were viewed in polarized light, any sample showing birefringence, and marked viscosity was taken to be liquid crystal.

(b) *Analytical method*

Mixtures of known composition were equilibrated for periods of not less than seven days in ampoules sealed by glass fusion and immersed in a water bath at 25°. The phases into which the solutions divided were separated by microsyringe, and analysed for three of the four components, the other being obtained by difference. Octanol, dodecane and C_8E_6 were determined by g.l.c. (Mulley & Winfield, 1970); the water content was measured by the Karl Fischer method.

(c) *Construction of the tetrahedral model*

The data obtained by the above methods were used to construct a three dimensional (Petrucci, 1965) tetrahedral model, using the geometric properties of this solid to represent the four-component system (Ricci, 1951). The faces (50 cm, sides) were made from 1/16 inch Perspex with chamfered edges for an accurate fit. Each heterogeneous region was built up from balsa wood, metal sheet and cellulose filler suitably painted, and could be dismantled to show the relation between the various regions.

RESULTS

The terms 1L, 2L, 1L + LC, 2L + LC and 3L represent one liquid, two-liquid, one liquid plus liquid crystal, two-liquid plus liquid crystal, and three-liquid, phases respectively. All composition data are given as percentage (w/w) of surface-active agent, octanol, water and dodecane in that order. Where uncertainty exists about the precise nature of phase equilibria within certain regions of the diagram e.g. in the case of the transition from 1L + LC to LC and vice versa, this is indicated by broken lines.

Binary and ternary systems

Fig. 1 shows a projection prepared by a method described by Woodman (1946) consisting of the four "peeled" ternary faces of the tetrahedron at 25°. A general view of the tetrahedral model is shown in Fig. 2. The system at 25° comprises two pairs of immiscible liquids (n-octanol–water, dodecane–water), the remaining four binary systems being completely miscible at this temperature. The binary surfactant–water system is shown in Fig. 3. Points A and D (Fig. 1) represent the solubility of octanol and dodecane in water, which have been reported as 0·03 at 20° (Marsden & Mann, 1963), and $8{\cdot}9 \times 10^{-10}$ mol fraction at 25° (Franks, 1966) respectively. Points C and F represent the solubility of water in octanol and dodecane; the former was found to be 3·52 at 25°, but the latter was not measured.

Phase analysis of conjugate layers in the main three component 2L areas (ABC, DEF, and ACFD Fig. 1) gave the direction of the tie-lines. These indicate that the plait points for the binodal curves ABC and DEF lie very close to the water and dodecane corners respectively. The results were also plotted as conjugation curves (Coolidge, 1926) and the surfactant content at the plait points was found in both cases to be below 1%. A further 2L region LMN exists at both 10 and 25°. Analytical investigation of its boundaries was made by preparing starting mixtures R and T and separating and analysing the phases, which then fixed the points R° and T° (Fig. 4).

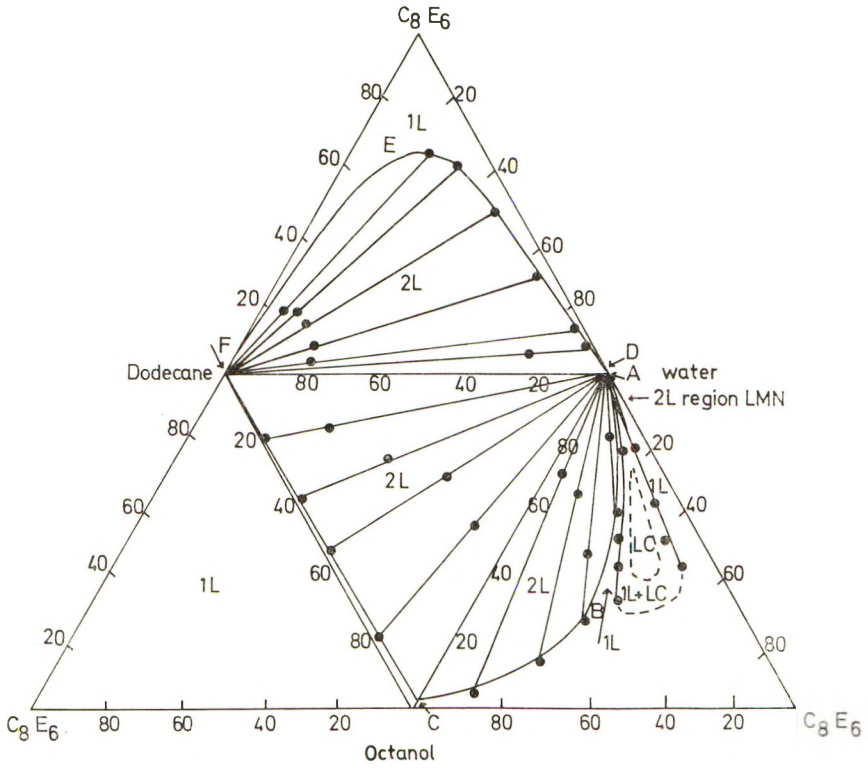

FIG. 1. Phase diagram of the four ternary faces of the tetrahedral model representing the system C_8E_6–water–octanol–dodecane at 25° plotted by Woodman's method (Woodman, 1946).

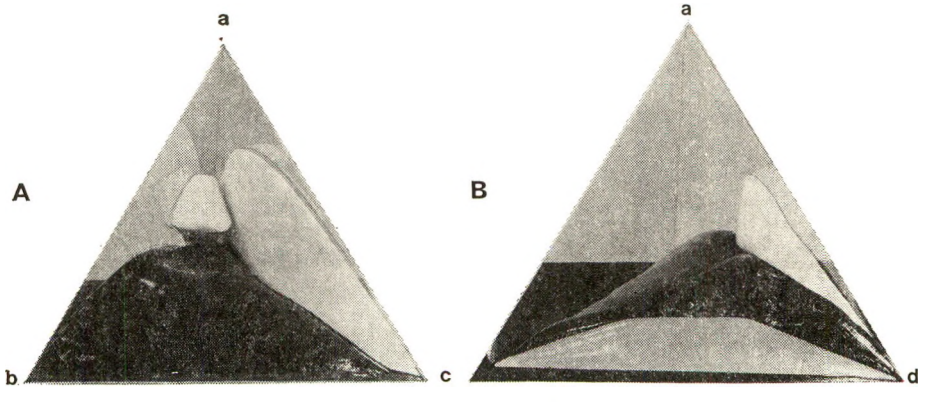

FIG. 2, A. Tetrahedral model of the system at 25° looking towards the C_8E_6–octanol–dodecane face. The 3L region may just be seen as a line between the black and the white 2L regions. The small light coloured volume extending from the octanol–water–C_8E_6 face is a liquid plus liquid crystal region.

B. Tetrahedral model looking towards the C_8E_6–dodecane–water face. The 2L volume extending from this face (the white region in Fig. 2A) has been removed to show the light coloured 3L region between this and the other 2L volume coming from the C_8E_6–octanol–water face. a, C_8E_6; b, octanol; c, dodecane; d, water.

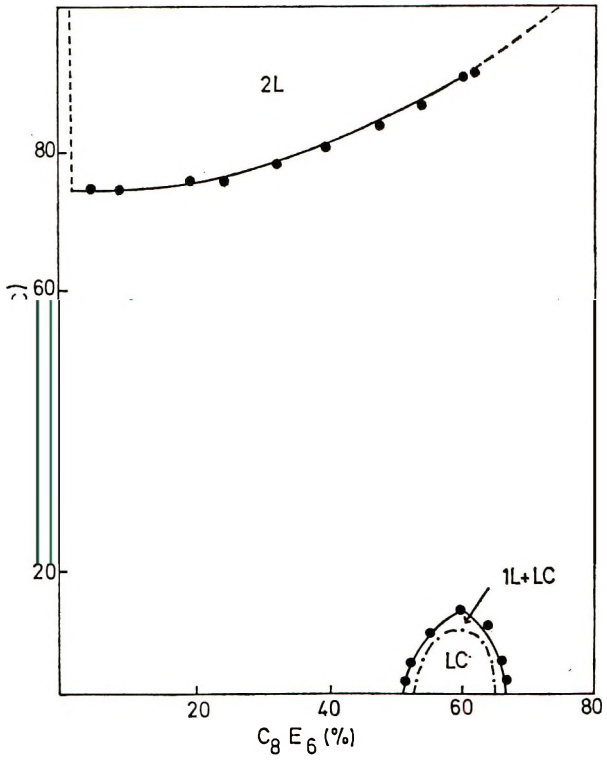

FIG. 3. Phase diagram for the system C_8E_6–water.

The area between the two 2L regions ABC and LMN was difficult to investigate mainly because small changes in composition markedly affected the observed phase situation. Difficulty was also experienced in equilibrating the systems. For example one mixture in this region (S, Fig. 4), after careful mixing and equilibration, separated into two liquids plus a liquid crystal phase, but after 14 days changed so that only one liquid and one liquid crystal phase were present. This was assumed to be the true situation, but the phase pattern within the region is shown by broken lines.

Quaternary system

Analytical data obtained in the 2L extension from the C_8E_6–octanol–water ternary face into the tetrahedron are given in Table 1, No. 3–23. The upper phase always contained most of the organic components including the surfactant, but the lower was almost entirely water, and contained so little of the other components that accurate analytical results were not obtained. In the 2L extension from the C_8E_6–water–dodecane ternary face, as in the ternary system, almost the exact reverse is the case; the lower aqueous phases are rich in surfactant and in equilibrium with upper ones containing very little. However, when the C_8E_6 content of the overall mixture is reduced, and substantial amounts of octanol are present, the composition of the upper phase begins to move away from the dodecane apex, and contains progressively more C_8E_6 and octanol (e.g. Table 1, No. 24 and 30). The tie-lines appear to 'swing over' without crossing. A similar situation must exist near the water axis for the other two-liquid region, but this was found impossible to demonstrate experimentally.

Table 1. *Tie line data in the two-liquid quaternary regions at* 25°. Results 1 and 2 lie in the volume extending from the ternary LMN region; 3–23 and 24–44 in the volumes extending from ABC and DEF respectively. A-(C_8E_6), B-(octanol), C-(dodecane). The water compositions may be obtained by difference.

	Total composition			Upper phase			Lower phase		
No	A	B	C	A	B	C	A	B	C
1	6·8	1·8	1·2	14	5	3·5			
2	4·55	1·5	1·7	15·5	4	5			
3	5·4	1·8	3·8	14·5	7	14			
4	10·1	2·9	1·7	17	4·5	2·5			
5	12·3	6·6	2·1	22	13·5	4·5			
6	6·35	3·4	2·65	25·5	14	11			
7	8·4	5·1	2·4	29	15·5	7·5	The lower phase		
8	6·2	3·2	0·8	13	6	1·5	was chiefly water		
9	4·9	14·9	47·5	7	22·5	69·5	and contained		
10	5·0	13·9	32·2	9	25	60	less than 0·5,		
11	5·15	31·5	19·1	9	51·5	35	0·2 and 0·3% of		
12	5·1	44·15	5·7	9	75	11·5	C_8E_6, octanol		
13	9·6	22·9	23·9	13	36·5	40	and dodecane		
14	9·6	13·5	36·9	14	21	53	respectively.		
15	16·7	26·3	17·2	20·5	36·5	24·5			
16	19·6	16·1	16·5	25·5	22	23			
17	20·1	22·5	7·0	27	33·5	9·5			
18	10·0	9·3	39·7	14	13·5	59			
19	11·7	12·2	69·3	14·5	14·5	53			
20	8·1	8·1	23·2	15	16	44·5			
21	7·4	8·75	42·3	12	14	64·5			
22	6·5	6·9	42·0	9	10	64·5			
23	9·35	8·7	40·8	11·5	13·5	66·5			
24	7·0	4·5	77·5	0·5	3·0	92·5	24·5	6	18·5
25	12·0	5·5	67·0	0·5	3·5	96	24·5	7	23
26	18·5	1·9	11·7	0·5	—	99·5	20	2	4
27	7·3	1·7	11·1	0·5	5·5	90	9	1	1·5
28	11·45	1·9	8·0	—	—	—	11·5	2·5	7
29	17·4	1·8	11·6	—	—	96·5	20	1·5	4·5
30	11·3	5·2	44·2	0·5	5	94	21	5·5	17·5
31	21·2	6·1	47·7	0·5	2·5	96	31	8	28·2
32	26·1	5·2	46·2	0·5	1·5	97	39	7	24·5
33	29·4	5·6	44·2	0·5	1·5	98	37	7	28
34	36·1	5·1	45·4	0·5	0·5	97	36·5	5	15
35	35·25	2·2	40·4	0·5	0·5	98·5	48	4	16·5
36	15·5	4·1	48·65	—	—	96	25·5	6	16
37	24·5	4·8	47·9	0·5	1	95·5	39·5	6	21·5
38	34·9	3·8	39·3	—	1	98·5	46	6	20·5
39	14·75	3·1	50·5	—	1	97·5	24	5·5	13
40	3	1·2	48·1	0·5	2	94·5	4·5	0·5	1·5
41	14·9	1·4	80·7	—	0·5	96	58	4	28
42	8·1	4·9	43·3	0·5	5·5	92	10·5	4·5	14·5
43	9·0	6·2	45·9	0·5	5·5	91·5	14·5	5	20·5
44	24·4	3·2	44·7	0·5	0·5	97	36·5	5	15

(—) results not obtained.

Tie-lines just interior to the ternary 2L region LMN of Fig. 4 follow the same pattern as in the extension of the 2L region ABC into the quaternary figure. Whether the two regions eventually join is difficult to say, but the main 2L region takes on a "humped" appearance in the vicinity of the water corner, which could be due to their amalgamation.

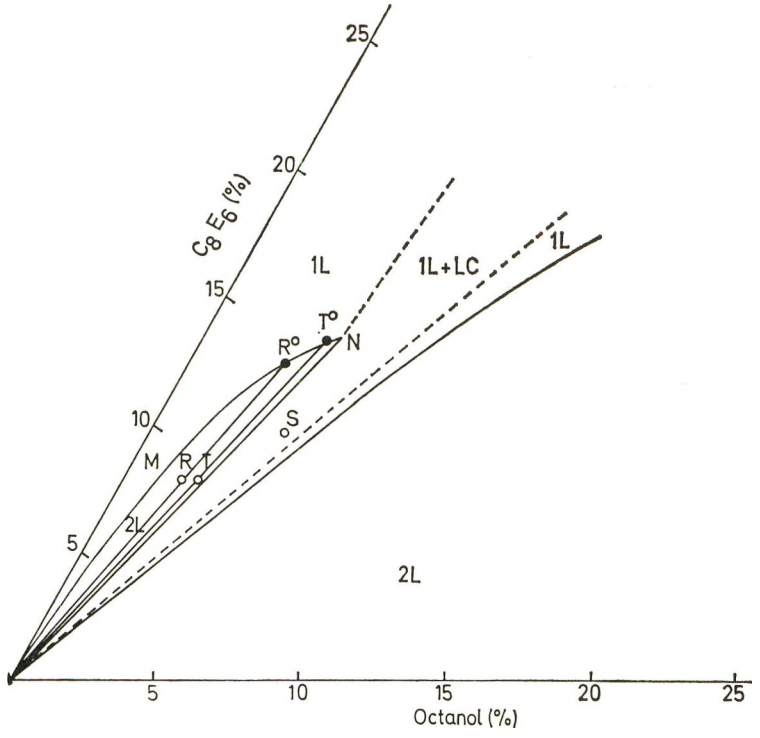

FIG. 4. The water-rich corner of the C_8E_6–octanol–water system at 25°.

3L region in the quaternary system

The position of this region is shown in Fig. 2B. In a quaternary system such a three-phase region has one degree of variance, unlike its counterpart in a three-component system. A composition in the three-phase region can be represented by a point in a triangular plane, the apices of which define the composition of each phase. The boundaries of the 3L region are defined by an infinite number of these planes. Lines joining their apices form edges to the region, and represent changes in the composition of the three phases concerned. The sides of the triangles form tie-lines in the surface between the 3L and 2L regions. Each set of data in Table 2 defines a triangular plane, and the projection drawn from these results (Fig. 5) gives an idea of the shape and size of the 3L region. The middle phase of the three contains a high percentage of non-ionic surfactant compared with the other two, and is also more variable in composition. The lower phase is essentially water, and experimental evidence for the changes in its composition, which must occur (see below), was not obtained. The upper phase consists of dodecane with small proportions of C_8E_6 and octanol; change in the composition of this phase, observed experimentally, is intermediate between that of the other two. The tie-line OP (Fig. 5) which represents the extremity of the 3L region in contact with the 2L volume above ACED, shows that the three liquid phases originate from the aqueous phase containing very small amounts of the other components (point O), and an oil phase, which is mainly dodecane (point P). As the overall amount of surfactant and octanol increases, the aqueous, oil, and third phase change in composition along the lines OX, PX, and OSX, and presumably end in a three-phase four-component plait point at X. The results define

Table 2. *Tie-line data in the three-liquid region at* 25°. A-(C_8E_6), B-(octanol), C-(dodecane). The water compositions may be obtained by difference.

Total composition			Upper phase			Middle phase		
A	B	C	A	B	C	A	B	C
5·9	6·55	44·8	2	8·5	88·5	13	13	49·5
9·1	6·5	43·6	2	8·5	88	15	6·5	25
3·3	4·3	45·9	2	8	88·5	5·5	2·5	9·5
4·75	4·9	47·4	2	7·5	89	7·5	3	11·5
8·6	6·3	42·1	—	7·5	88	15·5	6·5	26
7·9	6·6	42·6	—	—	—	13	10·5	46·5
5·5	6·1	45·1	2	9	88·5	14	8·5	31·5
9·7	6·5	43·4	—	7·5	92·5	13·5	8	30
9·2	9·1	60·9	4·5	9·5	72·5	13	10	55·5

(—) results not obtained. The third and lower phase was chiefly water and contained less than 0·5, 1·0 and 1·0% of C_8E_6, octanol and dodecane respectively.

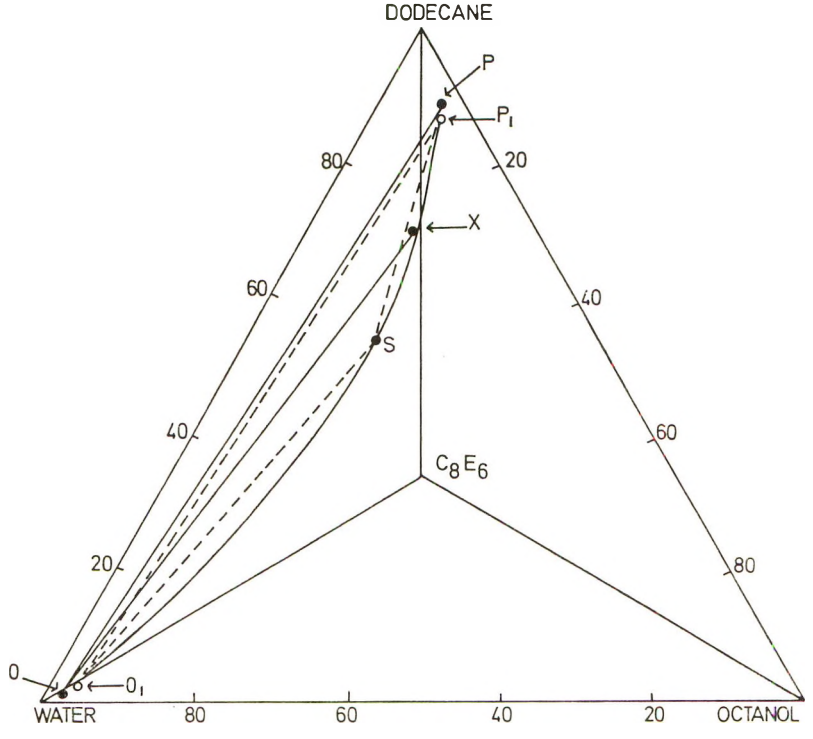

FIG. 5. Triangular-orthogonal projection of the three liquid region on the octanol–water–dodecane face. OX, PX and OSX represent the change in composition of the water, oil and surfactant phases respectively, OP being the last tie-line of the 3L region. O_1P_1S (broken lines) is a projection of plane 1, Table 2 (the position of points O, O_1 and P_1 are not shown to scale for clarity).

OSX and PX quite well, but as in the ternary system, the composition of the aqueous phase is very sensitive to changes in overall composition, which only occur in the immediate proximity of the line OX, and therefore, no data along OX were obtained.

Effect of temperature on the equilibria

The main difference between the ternary system C_8E_6–water–octanol at 10°, and the results at 25°, was a small increase in the 1L + LC region allied to a slight reduction in the area under the main 2L binodal curve. The liquid crystal region at both 10° and 25° extends from the vicinity of the water apex to concentrations of surfactant between 30 and 40%, but in the former instance the region appears to be rather wider. A further gel-like region appears on the ternary face C_8E_6–water–dodecane at 10°. This region, whilst not possessing any marked birefringence, had an appreciable viscosity, and, although not investigated thoroughly, appeared to form most readily at concentrations of surfactant and dodecane between 30 to 35% and 10 to 20% respectively. At 25°, it was only a little more viscous than a normal 1L phase, and it has therefore not been shown as a separate area in Fig. 1. In addition to the main gel or liquid crystal regions so far referred to, another smaller region extending from the water/C_8E_6 axis exists at 10°, which is presumably an extension of the liquid crystal region observed in the binary system at this temperature (Fig. 3).

In the quaternary system, results at 10° compared with those at 25°, revealed that the main liquid crystal region was more extensive at the former temperature. This was accompanied by a reduction in the 2L volume extending from the octanol–water–C_8E_6 face, and to a lesser extent in the 2L volume based on DEF (Fig. 1).

The synthetic data also showed the presence of a gel-like region at 10°, extending into the tetrahedron from the dodecane–water–C_8E_6 face, and lying very close to the 2L binodal curve. The gel was clear, and had a marked viscosity, but in most cases examined did not exhibit birefringence. Although the exact form of the diagram at 10° is uncertain, it seems unlikely that the gel merges with the main liquid crystal region. The area between the gel and the 2L region extending from the C_8E_6–water–dodecane face consisted of a white, opaque gel-like mass, which was obviously heterogeneous. Great difficulty was encountered in equilibrating the phases within this region, and no visible separation had occurred after seven days. Microscopy revealed the presence of two liquid phases, and it is probable that at least two liquids plus a liquid crystal phase are present.

DISCUSSION

Only a few quaternary systems have been investigated fully by phase-rule methods (Francis, 1963), and most of these were studied in connection with solvent extraction. A quaternary system derived from six pairs of liquids, four miscible and two immiscible, which also form a three-liquid phase region, does not appear to have been reported. In the present case the 3L region occurs near the ternary face which contains the two pairs of partial miscibility. It produces at first a single pair in the ternary and quaternary systems, but three phases occur when greater amounts of the fourth component are present. The nearest system we can find is that studied by Hartwig, Hood & Maycock (1955), which has three immiscible liquid pairs. Phase-rule studies on binary, ternary and a few quaternary systems containing surfactants have been made, mostly where the surfactant is ionic (McBain, 1950; Dervichian, 1957; Ekwall, Danielson & Mandell, 1960; Lawrence & Pearson, 1964), but a few on binary

and ternary systems containing non-ionic surfactants have also appeared (Marsden and McBain, 1948; Nakagawa & Tori, 1960; Mulley & Metcalfe, 1964; Shinoda, 1967; Corkill & Goodman, 1969; Friberg, Mandell & Fontell, 1969). These surfactant systems exhibit many differences from solvent mixtures, in particular the formation of liquid crystal phases.

From the former studies in surfactant systems it appears that the 1L phase near the C_8E_6–water axis, which extends over the 2L and 3L regions to the narrow single-phase organic solution bounded by CF and the octanol and dodecane apices (Figs 1 and 2), is a micellar solution of the two water-insoluble liquids, octanol and dodecane. The structure of organic solutions containing non-ionic surfactants has been less widely studied, but may contain micelles of the inverse type (Hartley, 1955). Solutions above CF may be like this. Solubility in the aqueous micellar solutions is limited in quaternary regions tending towards the C_8E_6–water–dodecane face, by separation of excess organic liquid (mainly dodecane), when more of the organic components are present than the micelles can hold. The tie-lines show the distribution of the surfactant between the phases to be unusual in comparison with that of other solutes in systems where the solute is miscible with both solvents. In most systems of the latter type the distribution is usually fairly even. This abnormality is repeated, although the distribution is reversed in the other main 2L region containing large amounts of octanol. Here the general form of the phase diagram is similar to the ternary system containing a phenol studied by Mulley & Metcalfe (1964), although in the present case a 1L "channel" (Palit, Moghe & Biswas, 1959) appears to separate the 2L region LMN and the liquid crystal area from the binodal curve ABC. The transition across the 1L/2L phase boundary ABC and its extension into the quaternary system, leads to an aqueous phase containing so little surfactant that micelles are probably not present, and the molecules are monomolecularly dispersed (Mulley & Metcalfe, 1964). Tie-line distributions of this type fit the theoretical considerations discussed by Mertslin & Nikurashina (1960) concerning 2L volumes surrounding three-phase regions in quaternary systems.

Formation of the 3L region between the two two-liquid volumes entering the tetrahedron from ABC and DEF, is the result of the different distribution of components within each, as shown by the two sets of tie-lines. Increasing the amounts of dodecane and water in the organic phase of the region extending from ABC, reduces the solubility of C_8E_6 so that eventually the phase divides into two: one rich in surfactant and containing some water forms, its composition given by a point on OSX; and the other containing an increasing amount of dodecane (composition along XP) (Fig. 5). The solution structure of 3L phases in such systems has been discussed earlier (Mulley & Metcalf, 1964). Further addition of dodecane–water moves the composition of the surfactant phase along XSO until eventually so little octanol is present that the surfactant and water phases merge at O, and the dodecane phase reaches P. Beyond this the characteristic distribution in the 2L volume extending from FED supervenes.

Difficulties found in measuring changes in composition of the aqueous phase were mentioned in the results section. Similar problems occur in other areas for the same reason. Included in these are the narrow channel region, and the phases situated in the surface near it, where the junction of the 3L and 2L phase regions are found. The latter are not separated in the base of the quaternary region, but it seems probable that a narrow 2L volume exists between and above the lines OSX and OX, although this was not confirmed experimentally.

The drop in temperature from 25 to 10° caused a reduction in volume of the main two- and three-liquid regions, although the general form of the diagrams is almost identical. This result is probably explained by a decrease in the hydrophilic nature of the surfactant at the higher temperature. The surfactant properties of the non-ionic class are almost destroyed above the cloud point. Rising temperature usually reduces liquid crystal formation by surfactants (McBain, 1950; Winfield, 1968), and this is the case in the present four-component system.

It is clear from this study that the surfactant, although miscible with water, octanol and dodecane, is distributed completely differently in the two pairs, water–octanol and water–dodecane, and that this will have important effects on the nature of emulsions formed within the two regions. The presence of three-liquid phases in some regions also means that the dispersions produced will be more complex than in two-phase emulsions. Liquid crystal, which is likely to be crucial in emulsion stability, does not invade the liquid phase regions in this surfactant system, but seems likely to do so with compounds containing longer alkyl chains.

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The kinetics of dissolution for a non-disintegrating standard substrate

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The dissolution profiles for a 1 cm sodium chloride cube, which was considered to represent a standard non-distintegrating substrate, were obtained in seven kinds of dissolution apparatus. The results were used to assess experimental reproducibility and apparatus variables and to examine the adherence of the kinetics of the dissolution process to theoretical rate laws.

The dissolution rate of a solid dosage form may be the rate limiting step in the pharmacodynamic processes controlling the physiological availability of a drug (see Wagner, 1961 for references).

Hixson & Crowell (1931) examined the dissolution kinetics for a solid, the surface area of which changed predictably with mass transfer into solution. Their "cube root law" was derived by assuming that: (*a*) dissolution takes place normal to the surface of the dissolving solid; (*b*) the same agitation effect is observed on all parts of the surface; (*c*) no stagnation of the liquid in any region within the volume of the solvent takes place; (*d*) solid particles remain intact throughout dissolution.

Meaningful dissolution kinetic coefficients, which are representative of the entire dissolution process, can be obtained only if the apparatus and the solid solute satisfy the stringent conditions laid down by Hixson & Crowell (1931). In addition, for any apparatus used to follow the progress of dissolution, dimensions, geometry and energy input should be rigidly specified if reproducible results are to be obtained.

The variation in physical properties between tablets which control dissolution, even from the same batch and lot number, is well recognized. Thus it has not been possible to differentiate between apparatus and vehicle (tablet) variables which may both contribute to observed variations in dissolution rate measurements for tablets with different formulations. In this paper a single cubic crystal of sodium chloride of one cm edge was used to distinguish intra and inter apparatus variables in seven kinds of apparatus. Dissolution profiles have been kinetically analysed and the conformation of the observed data from each apparatus to mathematical models has been tested.

MATERIALS AND METHODS

Apparatus

Seven types of dissolution apparatus were used. No attempt was made to examine the effect of changing variables like agitation intensity, solvent volume, geometry for a given apparatus. Similarly, test conditions were not selected to accentuate any advantages or disadvantages of a particular apparatus. Distilled water was used as solvent throughout. All dissolution studies were made at $37{\cdot}0 \pm 0{\cdot}1°$. Precise descriptions are given because designs permit some variation.

(1) *The stationary basket (S.B.)* (Fig. 1) described in part, by Cook, Chang & Mainville (1966) consisted of a 3-litre jar (Fisher, catalogue number 11–823) containing 2 litres of solvent, a T-shaped glass stirrer and a suspended basket constructed of stainless steel wire mesh. A sodium chloride cube was placed in the basket after thermal equilibrium had been achieved between the beaker, its contents and the temperature controlled bath. The stirrer was rotated clockwise (from above) at constant rate of 150 rev/min.

(2) *Food and Drug Directorate disintegration apparatus (F.D.D.)* (1965) (with the discs and plungers removed). A sodium chloride cube was placed in one of the cylinders of the tablet holder and the reciprocal motion of the latter was used to effect dissolution by agitation of 2·5 litres of solvent.

(3) *United States Pharmacopeia disintegration apparatus (U.S.P.).* The apparatus which has been described in detail elsewhere (Withey & Mainville, 1969), and method were similar to that described in (2) except that the volume of solvent was 800ml and that of the container was 1 litre as in the U.S.P. 1965.

(4) *The Levy Beaker (L.B.)* (Levy & Hollister, 1964). The three-bladed impeller was rotated clockwise (from above) at 60 rev/min and the volume of the solvent was 350 ml contained in a 400 ml beaker.

(5) *Resin kettle method (R.K.).* This was essentially the same as the apparatus described in (4) except that the container was a 1 litre resin kettle (Corning Glass Co., catalogue number 6947) and 900 ml of solvent was used.

(6) *Rotating basket method (R.B.).* Details are in the 1970 U.S.P. and N.F. The apparatus consisted of a cylindrical 40 mesh stainless steel wire basket (height 3·6 cm outside diameter, 2·5 cm) attached by spring clips to a 6 mm diameter stain-

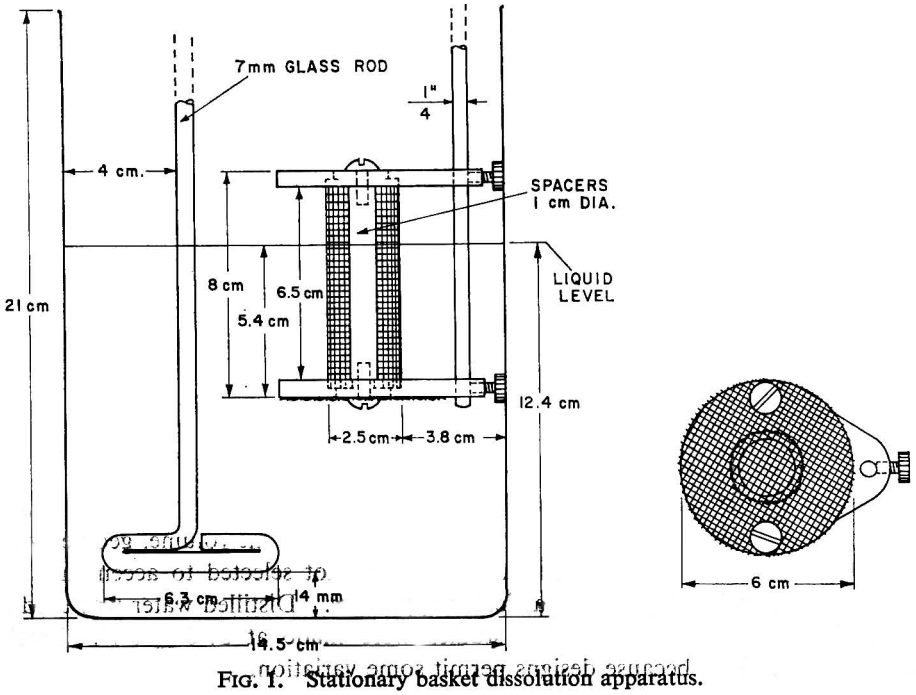

FIG. 1. Stationary basket dissolution apparatus.

less steel rod which fitted into a chuck attached to a constant speed motor (no impeller was used). A sodium chloride cube was placed in the basket which was then immersed in 900 ml of solvent, contained in a one litre resin kettle jar (as in 5 and rotated at 50 rev/min. The bottom of the wire mesh basket was 2 cm from the centre of the container bottom.

(7) *Tumbling cylinder method (T.C.)* (Withey & Mainville, 1969) (Fig. 2). This consisted of a lucite cylinder of equal height and internal diameter with a volume of 2 litres. A removable top (A) facilitated cleaning and the filling of the cylinder with solvent and a subsidiary access port (B) allowed the rapid insertion of the solid being examined. A liquid seal was effected by means of a 6 inch i.d. "O" ring (D). The cylinder was mounted in a U-shaped jig (C) and rotated about an axis at right angles to its height by means of a motor and drive shaft attached to the mid-point of the cylinder length. A second fixed, hollow support shaft (E) mounted opposite to the drive shaft was connected to the cylinder by means of a rotary seal[1] fabricated of stainless steel. In this study an electrolytic conductivity cell, G, was inserted through the hollow shaft and positioned in the body of the solvent. The whole apparatus was immersed in a bath at 37·0 ± 0·001°.

The rate of rotation of the cylinder (60 rev/min) was chosen because the solid remained intact and was bathed in solvent on all sides throughout the dissolution process. At <20 rev/min the cube rolled around the inside surface of the cylinder and at >100 rev/min the motion of the cube was erratic and unpredictable.

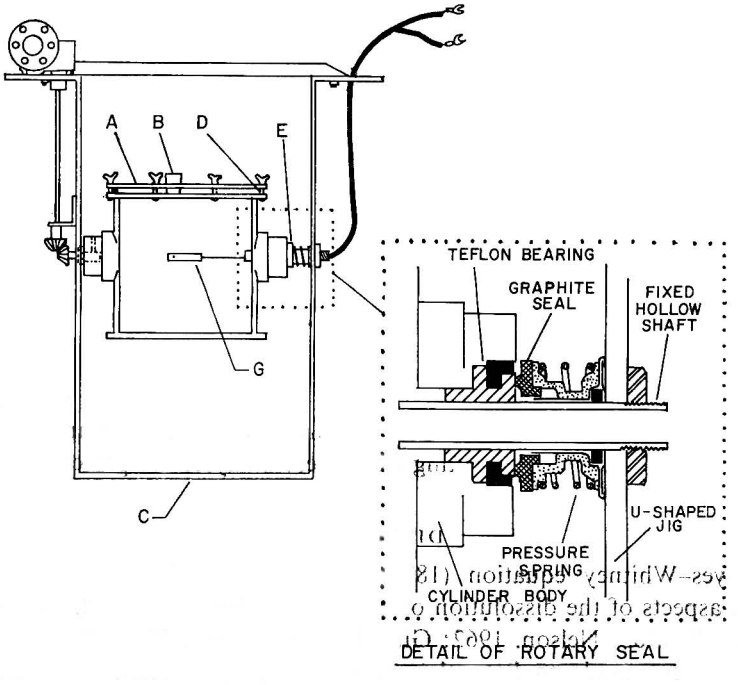

FIG. 2. Tumbling cylinder dissolution apparatus (labels refer to description which is given in the text).

[1] Beatty Pump Rotary Seal, catalogue No. 2000, Beatty Pumps, Chicago, Ill.

The sodium chloride cube of 1 cm edge with faces cut along the 100 axes[2] used for the test was considered an ideal non-disintegrating substrate. The mean and standard deviation of the weights of 50 of these were 2·5681 ± 0·350 g. The dissolution of a single cube was followed in each apparatus with four replicates by measuring the specific electrical conductivity of the solution using a platinum electrode probe[3] of cell constant 1·00 in the solution and connected to an automatic conductivity bridge[4]. As the temperature coefficient for the conductivity of salt solutions is large, the temperature was controlled to ±0·001° (Tronac[5] controller). The bridge was operated in a manual-hold condition which allowed the displayed conductance reading at any instant to be held. Initially readings were recorded every 5 s; for most runs about sixty readings were taken.

The apparatus giving the highest final concentration (i.e. having the smallest volume of solvent) was the Levy beaker (0·1056M) in which the dissolved cube gave a solution that was 17% saturated. Since the specific conductance of a solution of sodium chloride does not vary linearly with concentration above 1×10^{-3}M (Robertson, 1967), calibration curves of conductance against concentration between 1×10^{-3} and 150×10^{-3}M were constructed and the concentration of solutions were obtained from these by interpolation. The specific conductivity of solutions could

apparatus. The (% undissolved)$^{1/3}$ and log (% undissolved) mean curves are shown in Figs. 3 and 4. The means and standard deviations were also calculated after the logarithmic and cube root transformations had been applied. These calculations were done only for times at which each of the five runs had yielded data. To clarify the presentation every other datum point was omitted.

Two least squares lines were calculated for each of the 35 runs, one to the log (% undissolved) data and the other to the (% undissolved)$^{1/3}$ data. In these calculations it was assumed that the times were recorded without error and no weighting was introduced. Parameters resulting from these analyses are presented in Table 1.

DISCUSSION

The Noyes–Whitney equation (1897) has frequently been quoted where the theoretical aspects of the dissolution of a solid have been discussed. In some cases (Levy & Sahli, 1962; Nelson, 1962; Gibaldi & Weintraub, 1968; Tawashi, 1968) an attempt has been made to keep the surface area, S, constant in which case the

[2] Optovac, Inc., North Brookfield, Mass.
[3] Yellow Springs Instrument Corp., catalogue No. 3417.
[4] General Radio (Canada) Ltd., Toronto, type 1681, Impedance comparitor bridge.
[5] Type P.T.C.-1000, Tronac Inc., Provo, Utah, U.S.A.

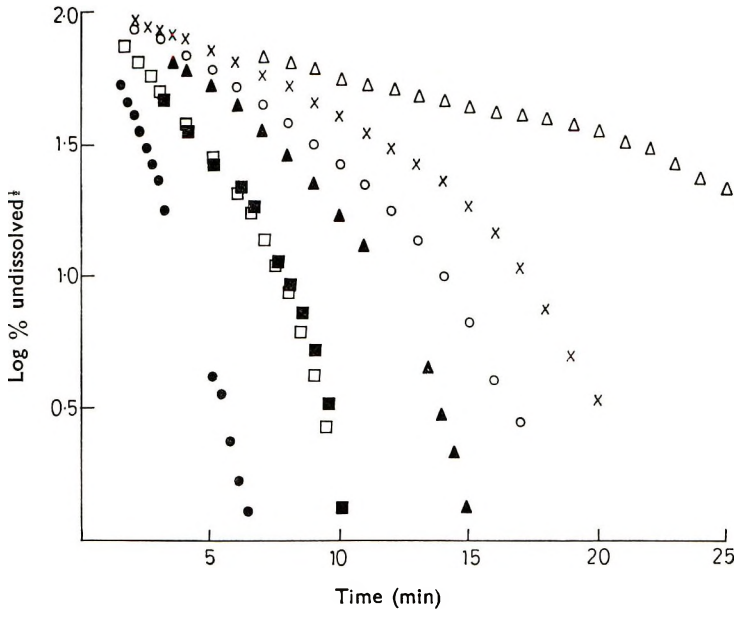

FIG. 3. Mean curves for the logarithm of % undissolved against time for the dissolution of a sodium chloride cube by different methods. × Levy beaker. ▲ stationary basket. △ Rotary basket. ■ F.D.D. □ U.S.P. ● Tumbling cylinder. ○ Resin kettle.

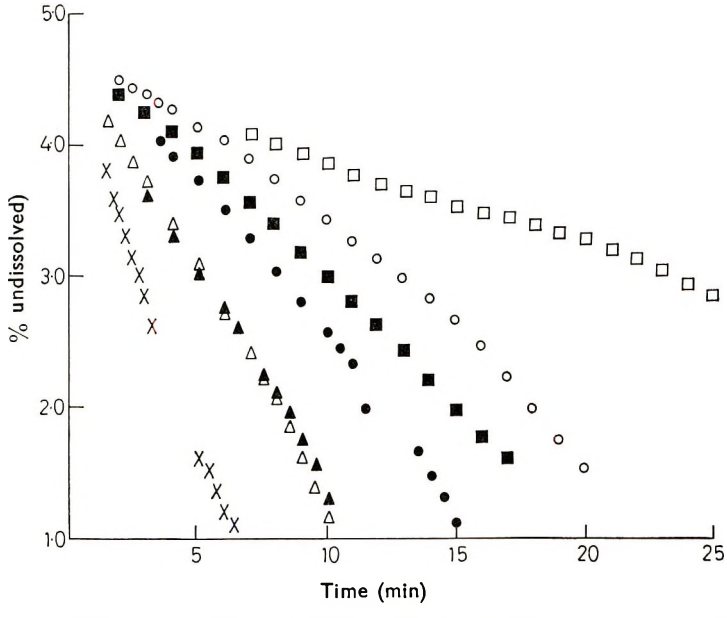

FIG. 4. Mean curves for the cube root of % undissolved against time for the dissolution of a sodium chloride cube by different methods. × Tumbling cylinder. ▲ F.D.D. △ U.S.P. ■ Resin kettle. □ Rotating basket. ● Stationary basket. ○ Levy beaker.

dissolution rate, in a sufficient volume of solvent to maintain sink conditions, becomes zero order. In other cases (Levy & Hollister, 1964), a first-order rate law has been assumed in the analysis of dissolution data although, as Wagner (1969) has pointed out, conformation of a dissolution process to a first-order law is usually an artifact.

It is usual to follow the dissolution of a solid by monitoring the concentration of substrate that appears in solution. Since the theoretical rate of dissolution of a solid is proportional to the area of the solid which is exposed to the solvent, it is necessary to predict the rate of change of surface area as the mass is transferred to solution.

For a perfect cube, of edge length x, the initial mass M_t, is equal to:

$$M_t = x^3 \rho \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where ρ is the density.

Thus, if M_0 is the initial mass then:

$$x = \left(\frac{M_0}{\rho}\right)^{1/3} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

The total surface area, S, of a cube is $6x^2$ so that:

$$S = 6\left(\frac{M_t}{\rho}\right)^{2/3} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

Substitution of (3) in the modified Noyes–Whitney equation (1897) yields the differential rate equation:

$$-\frac{dm}{dt} = 6k\, C_s \left(\frac{M_t}{\rho}\right)^{2/3} \quad .. \quad .. \quad .. \quad (4)$$

If M is the mass which remains undissolved at a time t, integration of (4) between the limits o and t gives:

$$\frac{3\rho^{2/3}}{6C_s}\left(M_0^{1/3} - M_t^{1/3}\right) = -kt \quad .. \quad .. \quad .. \quad (5)$$

which conforms to Hixson and Crowell's cube root dissolution law. A plot of $M_t^{1/3}$ against t should therefore give a linear relation of slope $-2C_sk/\rho^{2/3}$.

Three factors were considered in assessing the results of this investigation. These were: (*a*) Whether the observed dissolution rate followed an exponential (first-order) or cube root law; (*b*) The reproducibility of the results obtained by using a specific method and apparatus; (*c*) The relative order of magnitude of the overall rate of the dissolution process in a particular apparatus.

The data plotted on the cube root scale were found to be as or more linear than those plotted logarithmically (Figs 3 and 4) implying that the cube root law for the dissolution of a sodium chloride cube is more closely followed than the exponential first order law even for conditions which do not comply with the assumptions made by Hixson & Crowell (1931). It would appear that deviations of dissolution behaviour from a cube root law would better reflect formulation differences in a commercial tablet especially if Hixson and Crowell's conditions are met by the apparatus.

The foregoing conclusion is also supported by the analysis of individual curves which is presented in Table 1. The figures for the ratio of root mean squares r.m.s.

Table 1. *Derived parameters for data fitted to cube root and logarithmic functions.*

Apparatus	$y^{1/3} = a_1 + b_1x$			$\log y = a_2 + b_2x$			$\frac{\text{r.m.s.}_1}{\text{r.m.s.}_2}$	n
	a_1	$-b_1$	r.m.s.$_1$ $\times 10^2$	a_2	$-b_2 \times 10^2$	r.m.s.$_2$ $\times 10^3$		
Levy beaker ..	4·87	0·117	4·179	2·09	4·01	22·45	1·86	51
	4·85	0·139	3·242	2·09	4·87	20·22	1·60	48
	4·72	0·161	1·546	2·06	5·84	7·28	2·12	42
	4·78	0·135	3·726	2·07	4·76	22·63	1·65	49
	4·86	0·142	4·574	2·10	5·07	24·96	1·83	40
c.v.		11·5%						
Rotating basket	4·55	0·024	2·234	1·98	0·78	7·75	2·88	57
	4·50	0·069	6·776	1·99	2·55	17·44	3·89	57
	4·44	0·052	3·786	1·96	1·88	20·71	1·83	53
	4·63	0·071	3·102	2·04	2·59	13·04	2·38	39
	4·71	0·115	5·342	2·08	4·34	13·15	4·06	39
c.v.		49·7%						
Resin kettle ..	4·65	0·165	2·733	2·04	6·07	7·42	3·68	41
	4·96	0·176	1·826	2·14	6·39	13·67	1·34	18
	4·84	0·184	2·107	2·11	6·59	10·25	2·06	9
	4·86	0·172	3·918	2·12	6·31	19·39	2·02	17
	4·85	0·208	3·959	2·12	7·66	21·35	1·85	31
c.v.		9·0%						
Stationary basket	4·80	0·229	1·069	2·11	8·58	11·66	0·92	28
	4·91	0·214	1·704	2·15	7·97	13·34	1·28	21
	4·83	0·217	1·706	2·11	8·06	14·46	1·18	27
	4·81	0·226	1·400	2·12	8·73	12·61	1·11	27
	4·82	0·229	1·253	2·12	8·58	11·14	1·12	25
c.v.		3·2%						
F.D.D.	4·21	0·276	2·406	1·90	10·55	8·60	2·80	31
	4·26	0·276	3·398	1·92	10·75	10·68	3·18	30
	4·41	0·223	4·111	1·97	8·53	18·60	2·21	23
	4·59	0·343	2·319	2·04	13·14	6·63	3·50	23
	4·69	0·280	4·631	2·08	10·71	15·13	3·06	31
c.v.		15·2%						
U.S.P.	4·68	0·327	1·269	2·05	11·97	8·43	1·51	33
	4·68	0·328	1·972	2·05	11·86	9·27	2·13	33
	4·70	0·312	1·190	2·05	11·21	7·07	1·68	34
	4·68	0·317	2·572	2·06	11·79	6·16	4·18	32
	4·68	0·330	1·726	2·05	12·11	9·90	1·74	33
c.v.		2·4%						
Tumbling cylinder ..	4·61	0·599	5·220	2·07	23·98	16·73	3·12	20
	4·43	0·577	4·452	2·02	23·81	18·03	2·47	18
	4·70	0·597	2·099	2·11	23·97	8·72	2·41	22
	4·90	0·673	6·910	2·17	26·27	28·12	2·46	18
	4·60	0·544	3·122	2·08	21·94	12·33	2·53	25
c.v.		7·9%						

The following abbreviations are used in this table:

r.m.s. = Root mean square $\sqrt{(\text{observed} - \text{fitted})^2 / n-2}$

n = Number of observations used in the analysis,

c.v. = Coefficient of variation 100 $s/\bar{x}$, where s is the standard deviation.

of the cube root and log data ($r.m.s._1/r.m.s._2$) are generally smaller than the expected[6] range of between 2·1 and 3·6. The r.m.s. values for data of individual curves are compounded of a random component and a non-linear component (curvature). Part of the reason why the rms ratios fall below the calculated range is probably due to an unusually large curvature component in the logarithmic fit.

A comparison of the derived zero-time coefficients (a_1 and a_2 in Table 1) with the theoretical values of 4·64 for the cube root and 2·00 for the logarithmic function, shows little difference for the F.D.D. apparatus, the logarithmic coefficient to be closer for the rotating basket, and the cube root coefficient to be superior for the remainder.

Reproducibility within a given apparatus may be assessed from the coefficients of variation of the slope, b (Table 1). The magnitude of the coefficients of variation given in Table 1 indicate that the ranking in order of decreasing reproducibility was U.S.P., stationary basket, tumbling cylinder, Levy beaker, resin kettle, F.D.D. and rotating basket.

A comparison of the relative overall rates of dissolution for the different methods reveals that if the rate coefficient of dissolution is considered to be proportional to the slope of the cube root plot, then the ranking, in an order of increasing dissolution rate, is: rotating basket, Levy beaker, resin kettle, F.D.D., stationary basket, U.S.P. and tumbling cylinder.

If the sodium chloride cube is considered to be a standard substrate it is reasonable to conclude that this order is also the ranking for the agitation intensity of the dissolution medium in the various apparatus. The intensity of agitation has been considered as an important factor that can affect the rank order of dissolution rate for pharmaceutical dosage forms (Wurster & Taylor, 1965; Mitchell & Saville, 1969). No inter-apparatus comparisons of agitation intensity have been previously reported largely because of the variations in the method of agitation, geometry and design of apparatus components and volume of solvent. The same kind of agitation (a rotating three-bladed propeller) was used in both the Levy beaker and resin kettle methods and an identical reciprocating motion of the same disintegration basket was used in both the F.D.D. and U.S.P. methods. In both pairs, only the volume of solvent and the volume and shape of the containing vessel were different. The increase in volume of solvent from 800 ml to 2 litres in the U.S.P. and F.D.D. apparatus results in a decrease in dissolution rate of about 50%. Similarly an increase in solvent volume from 450 ml to 900 ml in the Levy beaker and resin kettle apparatus results in a decrease of 14·4% in the dissolution rate.

For the rotating basket apparatus, rotation of the basket in the solvent medium resulted in a very small vertical component to the agitation. When dissolution of

[6] The following argument was used to derive a range of expected values for the ratio of the root mean square ratio of the cube root and logarithmic data:

Let the difference between a measured point and the corresponding point on the theoretical curve be ε% Undissolved, a small enough amount to justify first order approximation.

Then, if we ignore the fact that the fitted curve is different in the two cases, the difference in the cube root transformed data will be:

$$(y + \varepsilon)^{1/3} - y$$

$$\sim y^{1/3}\,(\varepsilon/3y) \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

and in the logarithmically transformed data will be:

$$0{\cdot}434 \ln (y + \varepsilon) - \ln y$$

$$\frac{\sim 0{\cdot}434\varepsilon}{y} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

The r.m.s. ratio, assuming only small random errors, would be the ratio of equation (1) to (2) which is $0{\cdot}77y^{1/3}$. When = 20m the value is 2·1 and when y = 100, it is 3·6.

the cube commenced, the nearly saturated solution in the vicinity of the basket, remained near the bottom of the vessel owing to its relatively higher specific gravity. In subsequent determinations of dissolution rate the electrode was placed close to the upper surface of the solution but even this did not allow a true measurement of the intrinsic dissolution rate but rather monitored the sum of the dissolution rate of the cube and the rate of vertical mixing of the solution (or diffusion of solute molecules).

When the dissolution of a sodium chloride cube in the rotating basket apparatus was followed by a sampling technique in which three samples, one from near the bottom, one from the mid-point and one from near the surface of the solvent body, were abstracted simultaneously, the results depended on the depth of sampling (Fig. 5). Since most dissolution profiles for solid drug dosage forms are obtained by removing samples of the dissolution medium, spurious results could be obtained if its sampling depth is varied.

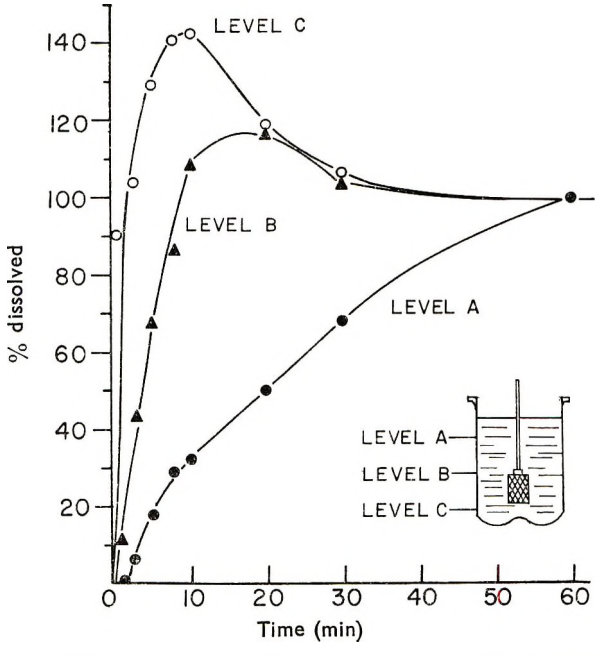

FIG. 5. Dissolution curves for a sodium chloride cube obtained by abstracting three samples simultaneously from the indicated levels.

CONCLUSIONS

It must be realized that few, if any, pharmaceutical dosage forms will behave like a sodium chloride cube in any of the apparatus since most preparations are formulated so that they disintegrate rapidly and the majority of drugs are probably not as soluble as sodium chloride. Although the U.S.P. and stationary basket apparatus allowed reproducible data to be obtained for the sodium chloride cube (and thus vindicated to some extent the use of the cube as a reference standard), the smaller particles of a disintegrated tablet would probably very rapidly become subject to less reproducible and more heterogeneous conditions after they had left their original

environment of a basket. In this respect, the tumbling cylinder may offer a more homogeneous and reproducible agitation of the dissolution medium. It should be mentioned that the dissolution rate was at least twice as fast in the tumbling cylinder as any other apparatus. This factor introduced some practical difficulties in assessing sampling times with sufficient precision since 80% of the dissolution had occurred in less than 2 min.

The Levy beaker and resin kettle apparatus, which have a close similarity, are probably more difficult to specify than any of the others in that the precise physical description of a three bladed impeller and its optimum rotation rate and geometry with respect to the containing vessel, preclude the reproduction of identical equipment. Sodium chloride cubes appeared (from visual observations) to dissolve, in the apparatus, in such a manner as to reduce the cube symmetry to that of a pyramid or cone.

The inherent liabilities of the rotating basket apparatus have been discussed in the light of the observation that, of the apparatus used in this study, the poorest reproducibility was obtained with this device.

Acknowledgements

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The influence of compression and formulation on the hardness, disintegration, dissolution, absorption and excretion of sulphadimidine tablets

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Sulphadimidine tablets were prepared with different binding agents and compressed at different compression levels, ranging from 200–2000 MNm^2. The disintegration time and dissolution rate of the different tablets were determined. Tablets formulated with gelatin or starch mucilage and compressed at 600 MNm^2 were selected for *in vivo* experiments using a urinary excretion method. Although both tablets complied with the disintegration requirements of the British Pharmacopoeia, dissolution rate and urinary excretion showed a difference in availability of drug from the two tablets.

Recently it was shown that a dissolution rate method in combination with an urinary excretion method can be used to evaluate sulphonamide tablets from different sources. With both methods a large difference in availability was found between sulphadimidine tablets from different sources. This was not so with sulphafurazole tablets, where all of the tablets had a rapid dissolution rate (Goossens & Van Oudtshoorn, 1969; van Oudtshoorn & Potgieter, 1970).

The dissolution rate of sulphamidine tablets has been examined by Smits & Nienhuis (1969), and the effect of dissolution rate from tablets on the absorption and excretion of sulphadimidine, using blood level data by Taraszka & Delor (1969). Dissolution rate methods were also used to study the effect of granulating agents on the dissolution time of sulphadimidine from tablets (Krowzynski & Stozek, 1968).

We have examined the effect of different compressional forces and formulation factors on the parameters used to characterize a particular sulphadimidine tablet formulation.

MATERIAL AND METHODS

Sulphadimidine powder, with an arithmatic mean particle size of 9·6 μm was used to prepare three different tablet formulations, I, II and III, each containing drug, 500 mg, starch 82 mg and magnesium stearate 6 mg. Formulation I was massed with methylcellulose (4 mg/tablet) (Tylose MH50), formulation II with 8% starch mucilage (12 mg/tablet) and formulation III with 8% gelatin mucilage (12 mg/tablet).

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Method of preparation. Formulation I was prepared according to the microgranulation technique of de Jong (1969). The sulphadimidine and starch were mixed in a plough-type mixer (Lödige) during 30 min. Methylcellulose was added as a 5% aqueous solution in three portions. The wet mass was passed through an 8 mm screen in a hammer mill (Apex). The microgranules were dried over 6 h at 35° and passed through a 310 μm screen.

Formulation II and III were prepared by mixing the sulphadimidine and starch in a planet type mixer while the mucilages of starch and gelatin respectively were added in small portions. Granulation was in a Manesty oscillating granulator with a 710 μm screen. The mass was dried at 35° over 16 h and passed through a 1200 μm screen. Before compression the three formulations were mixed with 5% starch and 1% magnesium stearate.

Compression. Tablets of 13·5 mm were compressed with flat punches on a single punch tablet machine, instrumented and calibrated as described by de Blaey and Polderman (1970). Six different compression levels varying from 200–2000 MNm^2 were chosen to obtain information over a wide compression range. The tablets were compressed once without ejection and a second time in order to calculate the work required to compress a particular formulation. The measurement was done by using force-displacement curves (de Blaey & Polderman, 1970). The hardness of the tablets was determined with a Heberlein hardness tester.

Assay method. The method of the British Pharmacopoeia (1968) was used for both the tablets and the powder.

Tablet disintegration. The tablet disintegration time was determined according to the British Pharmacopoeia (1968) using a Manesty tablet disintegration test unit with and without a disc.

Dissolution rate. Dissolution rate determinations were made using a beaker apparatus similar to that of Ganderton, Hadgraft & others (1967); 0·1N hydrochloric acid at 37° in a constant temperature bath was used as dissolution medium. The stirring rate was 100 rev/min. Samples were assayed continuously using an automated Bratton & Marshall (1939) method. The volume was kept constant by continuous addition of dissolution medium at 37°.

In vivo experiments. Tests were made on three healthy males between the ages of 22 and 28 who were slow acetylators of sulphadimidine (White & Evans, 1968). Two sulphadimidine tablets (1 g) were taken after an overnight fast and no food was taken for at least 1 h after. Quantitative urine collections were obtained at the times in Fig. 1. Aliquots of urine specimens were assayed according to Bratton & Marshall (1939) for free and total sulphadimidine. All hydrolysable conjugates were regarded as acetylated drug. The experiments took place under normal urine conditions. A digital computer program was used to calculate the rate and other constants according to the pharmacokinetic model and differential equations described by Nelson & O'Reilly (1960).

RESULTS AND DISCUSSION

Only two of the tablets from the tablet series (II and III compressed with an upper punch pressure of 200 MNm^2) would not have been acceptable according to the disintegration time requirements of the British Pharmacopoeia (1968) (Table 1). Their disintegration time with the disc was more than 15 min. When the time required for 50% of drug to go into solution (T50%) is also considered for II the

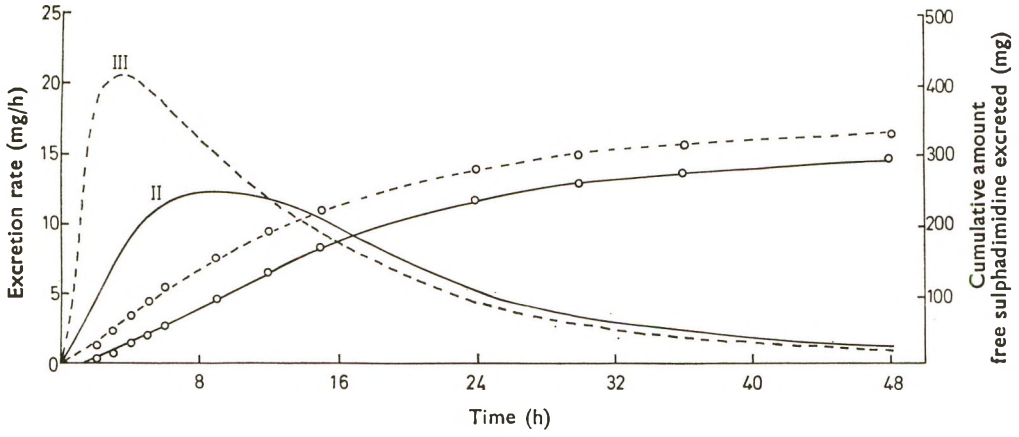

FIG. 1. Computed curves and experimental data points (○) for urinary excretion of free sulphadimidine from tablets II and III for test subject A under normal urine conditions.

dissolution time is much longer than the disintegration time and there is no correlation between dissolution rate and disintegration time as was shown by van Oudtshoorn & Potgieter (1970) for a series of sulphadimidine tablets.

Tablets of the three formulations compressed with an upper punch pressure of 600 MNm² were selected for further *in vitro* and *in vivo* tests because of the relatively short disintegration time, hardness and low friability. During the dissolution rate determinations it became obvious that tablet II had a much slower dissolution rate than might have been anticipated. Tablet I with the fastest disintegration time (0·91 min) also gave the best *in vitro* performance. This can be attributed to the microgranulation technique used and also to the hardness (8·5 kg) which is much lower than that of tablets II and III although compressional forces during manufacture were identical.

Because tablets II and III had approximately the same disintegration time, but different dissolution behaviour, they were selected for further *in vivo* experiments using a urinary excretion method. The results for one test subject are in Fig. 1. Both the cumulative amount of free sulphadimidine excreted as well as the excretion rate calculated from the slope of the cumulative amount excreted are given. The total amounts of sulphonamide excreted by the three test subjects over 48 h are shown in Table 2. From the figure for tablet II the dissolution rate can be seen to be a rate

Table 1. *Hardness, disintegration and dissolution of sulphadimidine tablets I, II and III.*

Upper punch pressure (MNm² ± 20)	Hardness* (kg) (Heberlein)			Disintegration time* (min) Without disc			With disc			Dissolution* (T50%, min)		
	I	II	III	I	II	III	I	II	III	I	II	III
200	3·0	5·2	5·5	0·68	>40·0	>40·0	—	>40·0	>40·0	0·5	54·0	10·0
400	5·5	8·0	11·5	0·68	>40·0	26·8	—	3·6	11·7	0·8	42·0	4·5
600	8·0	12·5	15·5	0·92	7·1	6·0	—	1·7	7·1	1·1	35·0	3·0
800	10·0	15·0	>16·0	1·07	4·1	8·0	—	1·3	6·6	1·2	10·0	4·6
1000	12·0	>16·0	>16·0	1·7	2·5	10·8	—	1·0	10·7	1·4	7·0	4·9
2000	15·0	>16·0	>16·0	4·00	2·3	12·4	—	2·1	10·8	1·8	3·3	6·5

* All figures are the average of three determinations.

Table 2. *Urinary excretion values (mg total sulphonamide) for three test subjects after ingestion of two sulphadimidine tablets (1·0g) II and III.*

Time (h)	Tablet II Test subject A	B	C	Tablet III Test subject A	B	C
1	1·75	2·15	2·41	10·25	15·60	14·31
2	8·76	10·10	10·43	40·62	49·10	47·57
3	21·10	23·85	25·05	82·67	92·94	86·50
4	38·42	43·05	43·31	126·30	137·97	125·73
5	61·26	66·81	68·15	171·04	184·31	163·55
6	87·61	94·39	96·47	207·84	224·35	197·94
9	183·25	193·73	198·09	334·50	355·98	318·47
12	285·17	301·58	304·82	448·65	473·28	422·09
15	372·30	391·90	393·34	539·01	562·70	510·9
24	545·56	560·86	563·77	715·62	718·95	670·22
36	635·95	645·69	654·35	803·95	804·27	760·65
48	668·70	676·48	682·33	832·09	834·78	787·63

limiting factor in the absorption process. The results are in agreement with those published earlier on sulphadimidine tablets of unknown composition (van Oudtshoorn & Potgieter, 1970).

During the dissolution experiments on tablet II it was noticed that a part of the tablet core was still intact on completion of the dissolution experiment. This might explain the low recovery of sulphadimidine in this particular *in vitro* and *in vivo* experiment. It is doubtful whether the difference observed between the two tablets would influence the therapeutic efficacy of the particular product to any extent. It does, however, show that an *in vitro* test procedure may be used to study the effects of formulation and process changes and to verify the physical quality of a product.

Acknowledgements

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The quantitative analysis of polyvinylpyrrolidone by infrared spectrophotometry

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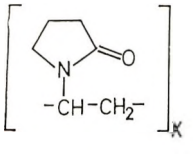
The quantitative assay of the binding agent polyvinylpyrrolidone (PVP) in granules of magnesium carbonate has been shown to be possible. The method is to determine the infrared absorption spectrum of a chloroform extract. Quantities of PVP down to 0·1 mg can be successfully assayed to $\pm 1\%$ in solution as dilute as 0·1% w/v.

Polyvinylpyrrolidone is a substance commonly included in granule formulations for its binding properties. However, because of its high molecular weight, its subsequent analysis particularly in combination with other ingredients has proved to be difficult. Despite the fact that its structural formula seems to include chromophoric groups (I), it exhibits no useful ultraviolet absorption peaks. Simonelli, Mehta & Higuchi (1969) have recently analysed PVP by measuring the total differential refractive indices of PVP solutions in a differential refractometer. This method, however, fails for dilute solutions, and almost any other soluble compound interferes.

PVP does, however, show two useful peaks in the middle infrared region. At 1460 cm^{-1} an absorption peak due to the scissor bending of $-CH_2$ groups occurs, but of particular interest is the high absorption peak at 1680 cm^{-1} due to the $>C=O$ groups. This peak has been used to assay PVP and the method has proved successful even in exceedingly dilute solutions.



I. Structural formula for polyvinylpyrrolidone. X = 180–6300.

The analytical method reported here was developed to enable the migration of PVP in the course of granule drying to be followed quantitatively. To do this, successive layers of powder were removed from the dried granules by controlled attrition. The powder removed was assayed for PVP content (Ridgway & Rubinstein, 1971).

METHODS

Spectra were obtained from a Unicam SP 100 infrared spectrophotometer working in the double-beam mode. The sample cell had potassium bromide windows and a fixed path length of 0·2 mm. The reference cell, also with potassium bromide windows, had a variable path length. Interference fringes were recorded for the empty sample cell, and the variable path length cell was adjusted to the same path length by observation of fringes. Chloroform (BDH Ltd., spectroscopic grade)

was injected into both cells, and spectra were run over the range 650–2150 cm^{-1}, checking that no absorption peaks were visible. A range of solutions of PVP (BDH Ltd., mol. wt. 44 000) were made up in the chloroform, and, using pure chloroform in the reference cell, spectra were obtained for each concentration.

Granules of magnesium carbonate were produced using known quantities of PVP as a binder. The method of production has been described elsewhere (Ridgway & Rubinstein, 1971). The granules were dried to constant weight in an oven, ground, weighed and shaken for 10 min with 5 ml of chloroform in a 10 ml stoppered flask. A sample was drawn into a syringe through a sweenex filter (Millipore Corp) fitted to the needle. The clear solution was then injected into the sample cell, and a spectrum obtained for each granule.

RESULTS AND DISCUSSION

The spectrum of PVP is shown in Fig. 1. The 'base line density' technique was used to evaluate the amount of PVP (Heigl, Bell & White, 1947). In this method a base line is drawn between two points selected, before and after the 1680 cm^{-1} peak, to give a line parallel as possible to the zero absorbance line.

The base line absorbance Ab is obtained from the equation

$$Ab = \log_{10} \frac{P_b}{P_o}$$

where P_b is the distance from the zero line to the base line, and P_o is the distance from the zero line to the top of the absorption peak.

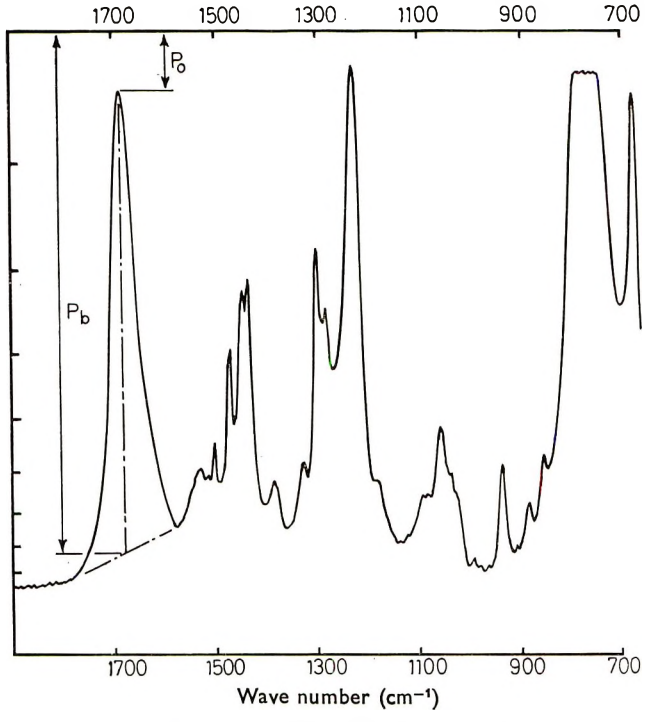


FIG. 1. The spectrum of polyvinylpyrrolidone (5% w/v in chloroform, path length 0·2 mm) in the infrared between 1800 and 650 cm^{-1}. The peak at 1680 cm^{-1} has been measured by the technique of Heigl, Bell & White (1947).

Both P_b and P_o were measured for the 1680 cm^{-1} line. A graph of absorbance (Ab) against PVP concentration was drawn for the prepared solutions of PVP whose concentration was known. A straight line resulted, showing that Beer's law applies over the concentration range 0·1–5% w/v. The gradient of the Beer's law line was 0·929 so that $\%\ \text{PVP, } \%\ \text{w/v} = \frac{\text{absorbance}}{0{\cdot}929}$. For the PVP solutions made by chloroform extraction of the granules, the absorbance was calculated and the corresponding concentration of PVP evaluated. From the solution volume and the weight of the granule the percentage of PVP in the granule could be calculated. This value could then be compared with the amount of PVP incorporated during their manufacture. Such a comparison is given below.

PVP (15 g) was used to granulate 290 g of magnesium carbonate, so that the PVP content was 4·91% by weight. Table 1 gives the results for six replicate analyses.

Table 1. *Results of six replicate analyses for ground and extracted $MgCO_3$ granules containing 4·91 wt % PVP.*

Wt of granular material in 5 ml chloroform	Absorbance of solution	% PVP w/v in solution	% PVP w/w in granule
1·23178	1·105	1·189	4·83
1·25473	1·147	1·235	4·92
1·26238	1·161	1·250	4·95
1·24923	1·101	1·185	4·82
1·26748	1·170	1·260	4·97
1·23942	1·119	1·205	4·86

Mean 4·89. Standard deviation = 0·06.

The mean value is close to the expected result, and the standard deviation is about 1%. The analytical method thus seems accurate; if the minimum quantity of PVP needed to make a solution of absorbance $\geqslant 0{\cdot}1$ were assayed, 0·1mg would be determinable to $\pm 1\%$.

Acknowledgement

We thank CIBA-Geigy (U.K.) Ltd. for the gift of the SP 100 infrared spectrometer.

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The mechanism of the inhibitory action of salicylate on glutamyl-transfer ribonucleic acid synthetase *in vitro*

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Salicylate inhibits glutamyl-transfer ribonucleic acid synthetase by simple competitive inhibition with respect to glutamate and mixed competitive-non-competitive inhibitions with ATP and tRNA. It is suggested that competition between salicylate and amino-acid explains the variable inhibition of the synthetases by the drugs *in vitro*. The action of salicylate is consistent with a mechanism for glutamyl-tRNA formation involving a concerted interaction of all three substrates. The drug may selectively interfere with protein synthesis *in vivo*.

Salicylate has been shown to inhibit the synthesis of aminoacyl-tRNAs (Burleigh & Smith, 1971). The most sensitive enzyme is glutamyl-tRNA synthetase. The present paper describes kinetic experiments designed to elucidate the mechanism of inhibition by using fixed concentrations of two of the substrates and varying the third in the presence of different concentrations of salicylate. Changes in the Mg^{2+}: ATP ratio in the reaction mixtures (Novelli, 1967) and the possible binding of salicylate to tRNA were also investigated; Burleigh & Smith (1970) gave a preliminary account.

MATERIALS AND METHODS

Materials

L-[U^{14}C]Glutamic acid (260 mCi/mmol) was obtained from the Radiochemical Centre, Amersham; ATP (sodium salt), L-glutamic acid, tRNA from Bakers yeast (Type III) and from calf liver (Type IV) and ribonuclease-A from bovine pancreas (salt-free) from the Sigma Chemical Company; PPO and POPOP from Packard Instrument Co. Inc; Sephadex G-25 from Pharmacia; Visking dialysis tubing (8/32 inch inflated diameter) from Scientific Instruments Centre, London.

Methods

The preparation of a 105 000 *g* supernatant and its passage through Sephadex G-25, the extraction of aminoacyl-tRNA, the measurement of radioactivity and the estimation of tRNA were according to Burleigh & Smith (1971).

Binding of salicylate to tRNA

This was studied by an equilibrium dialysis method (McArthur & Smith, 1969) using either 0·1M phosphate buffer (pH 7·4) or 0·1M tris-HCl (pH 7·4 at 20°) as dialysing medium. The Visking tubing was soaked in two changes of distilled water for 20 min before use. The tRNA solution (1 ml containing 500 μg of calf liver tRNA in buffer) was placed inside each dialysis sac at the beginning of the experiment and dialysed against 3 ml of buffer, containing initial salicylate concentrations ranging from 0–10 mM, for 24 h at 20° with shaking on a Luckman Rotary Shaker at 100

cycles/min. At the end of the experiment the unbound salicylate was measured in the fluids outside the dialysis sacs with an Aminco-Bowman spectrophotofluorometer at an excitation wavelength of 294 nm and a detection wavelength of 413 nm.

RESULTS

Kinetic experiments

In each experiment the activity of glutamyl-tRNA synthetase in the 105 000 *g* supernatant was estimated by measuring the incorporation of radioactivity from labelled glutamate into aminoacyl-tRNA at 2·0, 3·0 or 3·5 min at 37°. The reaction ceased to be linear after 4 min. The initial rates (v) were calculated from the slopes of the activity/time curves. Separate experiments were made with the three substrates; glutamate, ATP and tRNA, in which the concentrations of two of these were fixed and the third was varied in the presence of different concentrations of salicylate. Primary plots (1/v against 1/S) were constructed (Lineweaver & Burk, 1934) and the slopes from the primary plots were plotted against salicylate concentration.

Fig. 1 shows the results of varying the concentration of glutumate and salicylate in the presence of constant concentrations of ATP and tRNA. Figs 2 and 3 give the results of similar experiments in which the concentrations of salicylate and either ATP or tRNA were varied and those of glutamate and either tRNA or ATP were fixed.

The results in Fig. 1 show that the primary plots intersect on the 1/v axis and that the secondary plot of the slopes against salicylate concentration is linear. They are consistent with a mechanism of inhibition involving simple competition between

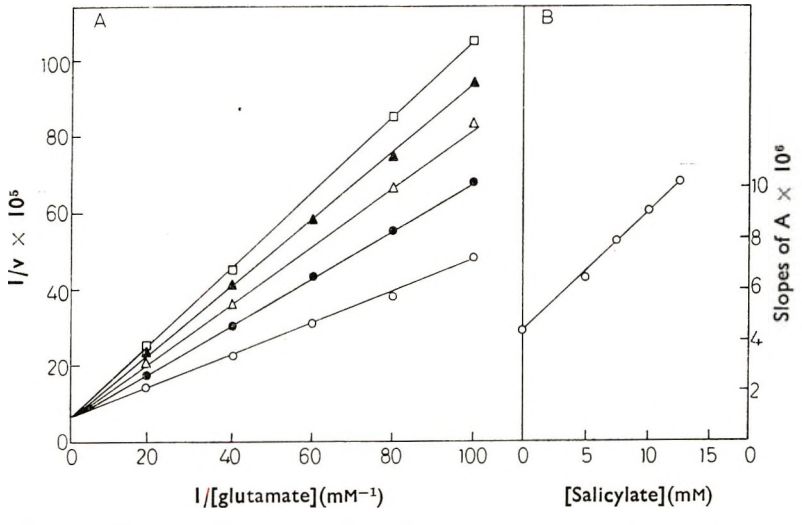
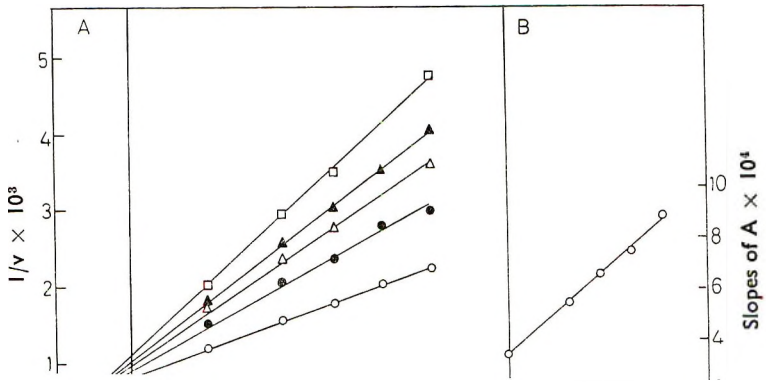


FIG. 1. Glutamyl-tRNA synthetase, kinetics with constant concentrations of ATP and tRNA. Each reaction mixture contained, in a total volume of 1·0 ml, 100 μmol tris-HCl (pH 7·2 at 37°); 10 μmol $MgCl_2$; 2 μmol ATP; 500 μg yeast tRNA; 0·4 ml 105 000 *g* supernatant (7·5 mg protein); sufficient of a mixture of KCl and potassium salicylate to yield final salicylate concentrations ranging from 0–12·5 mM and a constant K^+ concentration of 15 mM; and mixtures of L-[^{14}C]-glutamate and L-glutamate to give total glutamate concentrations ranging from 0·01 to 0·05 mM and specific activities from 200 to 20 mCi/mmol. The reaction was started by the addition of the supernatant and duplicate tubes were incubated at 37° for, 2, 3 or 3·5 min. Zero time counts have been subtracted from the experimental values which were calculated as counts/min mg^{-1} RNA isolated corrected to a specific activity of radioactive glutamate of 100 mCi/mmol. A. ○—○ Control; ●—● 5·0 mM salicylate; △—△ 7·5 mM salicylate; ▲—▲ 10·0 mM salicylate; □—□ 12·5 mM salicylate. B. Secondary plot of slopes of A.



One possible explanation of the inhibitory effect of salicylate on the synthetase activity is that the drug binds to the tRNA. The equilibrium dialysis experiments revealed that no such binding was observed in a tris-HCl medium and a similar result was obtained with the phosphate medium.

DISCUSSION

The results show that salicylate inhibits the activity of glutamyl-tRNA synthetase by a simple competitive mechanism involving glutamate and mixed competitive-non-competitive mechanisms with ATP and tRNA. The drug did not act either by altering the effective concentration of Mg^{2+} or by binding to the tRNA.

There are several implications of the results. One concerns the mechanism for the biosynthesis of aminoacyl-tRNA. This has been suggested to occur in two stages, activation of the amino-acids (Hoagland, 1955) and their transfer to the tRNA (Hoagland, Zamecnik & Stephenson, 1957). Equations 1 and 2 show the inter-

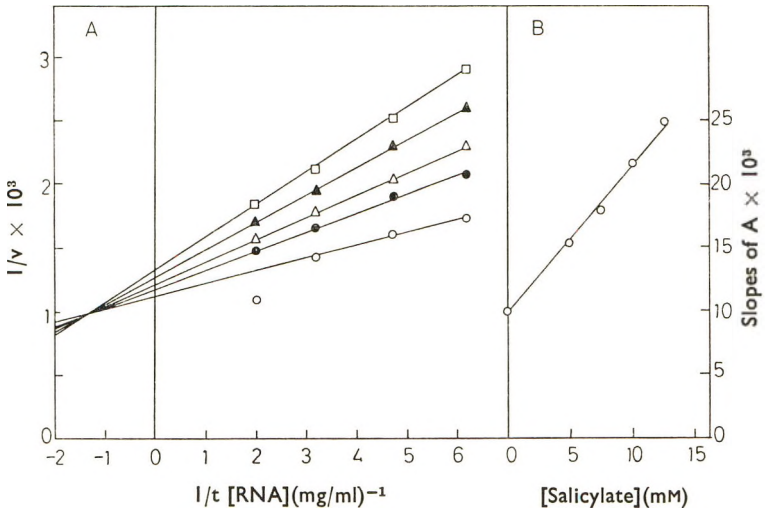


FIG. 3. Glutamyl-tRNA synthetase, kinetics with constant concentrations of glutamate and ATP. Experimental conditions similar to Fig. 2 except that the concentration of ATP in each reaction mixture was 2 mM and the yeast tRNA was replaced by sufficient calf liver tRNA to give final tRNA concentrations (calf + endogenous) ranging from 162–504 μg/ml. At the end of the incubation sufficient calf liver tRNA was added to give a total of 1 mg per tube. A. ○—○ Control; ●—● 5·0 mM salicylate; △—△ 7·5 mM salicylate; ▲—▲ 10·0 mM salicylate; □—□ 12·5 mM salicylate. B. Secondary plot of slopes of A.

mediates involved and equation 3 represents the over-all reaction. The symbols refer to the following: AA is amino-acid, PP_i is pyrophosphate, E is enzyme, E(AA — AMP) is enzyme-bound aminoacyl-adenylate and AA-tRNA is aminoacyl-transfer ribonucleic acid.

$$AA + ATP + E \rightleftharpoons E(AA - AMP) + PP_i \quad .. \quad .. \quad (1)$$

$$E(AA - AMP) + tRNA \rightleftharpoons AA - tRNA + AMP + E \quad .. \quad (2)$$

$$AA + ATP + tRNA \rightleftharpoons AA - tRNA + AMP + PP_i \quad .. \quad (3)$$

The main evidence in favour of a two-stage mechanism is the isolation of radioactive enzyme-bound aminoacyl-adenylates from reaction mixtures containing ATP, substrate amounts of purified enzymes from either bacteria or mammalian tissues and either [^{14}C]isoleucine, [^{14}C]tryptophan or [^{14}C]threonine (Wong & Moldave, 1960; Norris & Berg, 1964; Allende, Allende & others, 1964). The results of experiments using carboxy-[^{18}O]tryptophan (Hoagland, Zamecnik & others, 1957) are consistent with the two-stage mechanism but do not exclude a single concerted reaction as in equation 3 (Boyer, 1960). The ATP-pyrophosphate exchange reaction has been considered to reflect equation 1 but it has been observed that in mammalian systems glutamate does not promote this exchange in the absence of tRNA (Deutscher, 1967) and a similar result occurs with several amino-acids and bacterial aminoacyl-tRNA synthetases (Mitra & Mehler, 1967). The participation of E(AA — AMP) as an obligatory intermediate was questioned by Loftfield & Eigner (1969) on the basis of their data on the formation of aminoacyl hydroxamates. These workers (Loftfield & Eigner, 1968) also showed that ammonia and imidazole inhibit the ATP-pyrophosphate exchange but stimulate the esterification of tRNA. Salicylate inhibits the exchange reaction promoted by leucine and phenylalanine but does not affect the incorporation of these amino-acids into aminoacyl-tRNA (Burleigh & Smith, 1971).

The results of the present work (Figs 1–3) are consistent only with a mechanism for the action of glutamyl-tRNA synthetase involving a concerted interaction of the three substrates leading to the formation of a quaternary complex. A two-stage reaction mechanism would necessitate the inhibition by salicylate being uncompetitive with respect to tRNA (Fromm, 1967). Although our work has been restricted to the glutamate enzyme similar considerations could apply to the other aminoacyl-tRNA synthetases. The formation of enzyme-bound aminoacyl-adenylates from certain amino-acids, reflected by the ATP-pyrophosphate exchange, may occur as a side reaction.

The proposed mechanism of inhibition of the aminoacyl-tRNA synthetases by salicylate, involving simple competition with the amino-acid substrates, suggest that the drug may selectively interfere with protein biosynthesis *in vivo*. Whether or not the incorporation of a particular amino-acid into tissue proteins were inhibited would depend on the endogenous concentration of the amino-acid, the K_m of the corresponding synthetase and the K_I of the enzyme for salicylate.

Some proteins are relatively rich in certain amino-acids. An example is the immunoglobulins which contain more tryptophan than other proteins (see Press & Piggot, 1967). If the incorporation of tryptophan plays a part in regulating the biosynthesis of immunoglobulins *in vivo* then salicylate may act as a selective inhibitor by affecting tissues, such as lymphocytes, which are principally concerned with the manufacture of antibodies. A further possibility in the same category is pyrrolidone-γ-carboxylate, which is a common *N*-terminal group in antibodies and may also act as an initiator of immunoglobulin biosynthesis (Wikler, Titani & others, 1967).

Acknowledgements

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The neuromuscular blocking activity of some monoquaternary androstane derivatives

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The neuromuscular blocking activity of some 3- or 17-monoquaternary derivatives of 3β,17β-dipyrrolidin-1′-yl-5α-androstane has been examined in the cat. 3-Monoquaternary salts were, in general, more potent than the 17-monoquaternary salts. It was concluded that the presence of a quaternary group in position 3 is an important determinant of potency in this series of androstane derivatives.

3β,17β-Dipyrrolidin-1′-yl-5α-androstane bismethochloride (I) (dipyrandium chloride) is a potent, non-depolarizing curarizing agent both in animals and in man (Biggs, Davis & Wien, 1964; Mushin & Mapleson, 1964; Lees & Tavernor, 1969). This compound is one of eight possible stereiosomers, the α- and β-epimers at each of positions 3, 5 and 17. A study of these isomers (Bamford, Biggs & others, 1967) showed that the 3β-compounds were always more potent than the corresponding 3α-isomers, and that inversion of the basic centre at position 17 had little effect on potency. This suggested to us that in the interaction with the receptor, position 3 was more important in determining potency in this series of steroidal neuromuscular blocking agents than position 17. To test this hypothesis we synthesised several monoquaternary salts of 3- or 17-pyrrolidin-1′-yl-5α-androstane, including the 3β-(III) and 17β-monoquaternary salts (IV) corresponding to dipyrandium chloride, and compared their potency with dipyrandium chloride and with the corresponding unquaternized dihydrochloride (II) (Davis, Parnell & Rosenbaum, 1967).

CHEMISTRY

The synthesis of 3β,17β-dipyrrolidin-1′-yl-5α-androstane (II) and its bisquaternary salts has been described by Davis & others (1967).

A study of the reaction of 3β,17β-dipyrrolidin-1′-yl-5α-androstane with methyl iodide showed that quaternization at position 3 was rapid, and subsequent conversion into the 3,17-bisquaternary salt much slower. Brief heating of the compound with methyl iodide thus gave in good yield the 3-methiodide (III, X = I), which was converted by ion-exchange into the more soluble methochloride (III, X = Cl).

The 3-methiodides (V) and (VII) were prepared by treatment of the known corresponding tertiary bases (Davis, Parnell & Rosenbaum, 1966) with methyl iodide in methanol.

The 17β-monoquaternary salt (IV) was prepared by a Leuckart-Wallach reaction, which is known to be stereospecific, from 17β-pyrrolidin-1′-yl-5α-androstan-3-one methiodide (VI) (Davis, Parnell & Warburton, 1966a). The same authors have described the salt (VIII).

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17β-Pyrrolidin-1′-yl-5α-androstane was also obtained by the Leuckart-Wallach method from 5α-androstan-17-one (Brutcher & Bauer, 1962), and was quaternized to the iodide (IX, X = I) in the usual way. Its isomer, 17α-pyrrolidin-1′-yl-5α-androstane, was prepared from 17β-tosyloxy-5α-androstane (Elks & Shoppee, 1953) by inversion at position 17 with pyrrolidine (Davis & others, 1966), and likewise gave the methiodide (X, X = I). For pharmacological testing these two methiodides were converted into the soluble methochlorides.

PHARMACOLOGY

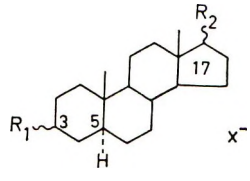
Relative molar potency was determined *in vivo* on the cat sciatic nerve–tibialis muscle preparation using the method described by Bamford & others (1967).

RESULTS AND DISCUSSION

The results have been summarized in Table 1. All the compounds had a non-depolarizing mechanism of action as shown by the reversal of blockade by edrophonium (0·5 mg/kg) intravenously and the flaccid paralysis they caused when injected into day-old chicks. All compounds had a similar duration of action in the cat.

A comparison of the results for compounds III and IV, and for V and VI showed that the 3β-monoquaternary salts were more potent than the corresponding 17β-salts. This would be predicted from the study of the stereoisomers of the parent compound, dipyrandium chloride (I). A possible exception to this was seen with compounds VII and VIII, which had approximately equal activity, but only of a low order. In addition, comparison of compounds III, V and VII with dipyrandium

Table 1. *The relative neuromuscular blocking activities of* 3β,17β-*dipyrrolidin*-1′-*yl*-5α-*androstane bismethiodide* (I) *and some related monoquaternary salts in the cat.*



Compound No.	R_2	R_3	X^-	Relative Molar Potency*
I	β-1-Methyl-1-pyrrolidin-1-yl	β-1-Methyl-1-pyrrolidin-1-yl	$2Cl^-$	1·00
II	β-Pyrrolidin-1-yl	β-Pyrrolidin-1-yl	$2Cl^-$	0·04
III	β-1-Methyl-1-pyrrolidin-1-yl	β-Pyrrolidin-1-yl	$2Cl^-$	1·07
IV	β-Pyrrolidin-1-yl	β-1-Methyl-1-pyrrolidin-1-yl	$2I^-$	0·22
V	β-1-Methyl-1-pyrrolidin-1-yl	= O	I^-	0·025
VI	= O	β-1-Methyl-1-pyrrolidin-1-yl	I^-	0·0013
VII	β-1-Methyl-1-pyrrolidin-1-yl	β-Hydroxy	I^-	0·0066
VIII	β-Hydroxy	β-1-Methyl-1-pyrrolidin-1-yl	I^-	0·0081
IX	-H	β-1-Methyl-1-pyrrolidin-1-yl	Cl^-	<0·007
X	-H	α-1-Methyl-1-pyrrolidin-1-yl	Cl^-	<0·007
	(+)-Tubocurarine			1·31
	Gallamine triethiodide			0·19

* Each figure represents the mean of two or more determinations.

chloride demonstrates that, in this series of 3β-quaternary salts, potency is maximal when there is also a basic group in position 17, whether quaternized or not, and is reduced by a factor of 40–150 when there is a 17-keto- or 17β-hydroxy-group.

As would be expected the epimeric compounds IX and X lacking any basic centre in position 3, were both inactive.

Since this work was completed, Lewis, Martin-Smith & others (1967) have reported some 2β- and 3α-monoquaternary ammonium salts in the androstane and pregnane series. All their compounds displayed typical non-depolarizing activity but were of low potency, the most active of the 3α-quaternary salts being 2β,17β-diacetoxy-3α-piperidino-5α-androstane methobromide, with 0·02 of the relative molar potency of (+)-tubocurarine in the cat.

More recently Busfield, Child & others (1968) have examined quaternary salts of the steroidal alkaloid conessine, and have found several 3β-monoquaternary compounds to be potent drugs, producing a typical curare-like response of short duration.

These results support the one-point-attachment theory and our hypothesis that the quaternary centre in position 3 is more important than one in position 17 in determining potency in this series.

METHODS

Quaternization of 3β,17β-*dipyrrolidin*-1′-*yl*-5α-*androstane*. A mixture of the ditertiary base (0·3 g), methyl iodide (0·3 ml) and methanol (3 ml) was heated under reflux. Samples (0·1 ml) were withdrawn at intervals and evaporated immediately to dryness, and the residues were dissolved in ethanol–chloroform and applied to an alumina-coated chromato-plate. Development was with ethanol–chloroform–ethyl acetate–water–concentrated hydrochloric acid (60:60:60:8:3), and the developed chromatogram was sprayed with Dragendorff reagent.

The results shows that the ditertiary base (R_F 0·6) rapidly disappeared and only a trace was left after 10 min. At this time the proportion of the 3-methiodide (R_F 0·3) had reached a peak, with only a trace of the 3,17-bismethiodide (R_F 0·1) present. Subsequent conversion of the mono- into the bis-quaternary salt was much slower and was not quite complete when the experiment was terminated after 320 min.

3β,17β-*Dipyrrolidin*-1′*yl*-5α-*androstane* 3-*methochloride hydrochloride* (III). A mixture of 3β,17β-dipyrrolidin-1′-yl-5α-androstane (0·7 g), methyl iodide (0·7 ml) and methanol (7 ml) was heated under reflux for 10 min, and then evaporated to dryness. The residue was boiled with benzene (25 ml) to remove unchanged base, and the insoluble salt was crystallized from aqueous methanol, giving the 3-*methiodide* (0·6 g), m.p. $>300°$ (darkens from 265°). (Found: I, 24·0; N, 5·0. $C_{28}H_{49}IN_2$ requires I, 23·5; N, 5·2%).

A solution of the methiodide (0·45 g) in 50% aqueous methanol (25 ml) was percolated through a column of De-Acidite FF ion-exchange resin (5 ml, chloride form) and washed through with a further quantity (10 ml) of the solvent. The combined eluates were acidified with hydrochloric acid and evaporated. The residue was crystallized from ethanol–ethyl acetate, yielding the 3-*methochloride hydrochloride* (0·32 g), m.p. $>300°$. (Found: Cl, 14·2; N, 5·5. $C_{28}H_{49}ClN_2$, HCl requires Cl, 14·7; N, 5·8%).

3β,17β-*Dipyrrolidin*-1′-*yl*-5α-*androstane* 17-*methiodide hydriodide* (IV). A mixture of 17β-pyrrolidin-1′-yl-5α-androstan-3-one methiodide (1·6 g), pyrrolidine (2 ml) and

formic acid (2 ml) was heated under reflux at 160–170° for 16 h, cooled and diluted with ethyl acetate. The solid product was dissolved in a mixture of water (15 ml) and concentrated hydrochloric acid (1 ml) and sodium iodide (2 g) was added to the filtered solution. The product was filtered off and washed with hot acetone, then recrystallized from water, giving the 17-*methiodide hydriodide* (0·4 g), m.p. >300°. (Found: C, 50·5; H, 7·5; I, 37·7. $C_{28}H_{49}IN_2$,HI requires C, 50·3; H, 7·5; I, 37·95%).

3β-*Pyrrolidin*-1′-*yl*-5α-*androstan*-17-*one methiodide* (V). 3β-Pyrrolidin-1′-yl-5α-androstan-17-one (2·0 g) was dissolved in cold methyl iodide (10 ml). An exothermic reaction took place and a crystalline solid separated. The suspension was heated under reflux for 20 min, diluted with an excess of ethyl acetate, cooled and filtered. The solid was washed with ethyl acetate, dissolved in a mixture of methanol (15 ml) and ethyl acetate (45 ml) and the hot mixture was decolorized with charcoal and filtered through "Hyflo Supercel". Concentration of the filtrate and cooling afforded 3β-*pyrrolidin*-1′-*yl*-5α-*androstan*-17-*one methiodide* (2·5 g, 88%), m.p. 268–269°. (Found: I, 25·9. $C_{24}H_{40}INO$ requires I, 26·1%).

Similarly prepared was 3β-*pyrrolidin*-1′-*yl*-5α-*androstan*-17β-*ol methiodide* (VII) (92%), m.p. 292–294° (decomp). (Found: I, 25·6. $C_{24}H_{42}INO$ requires I, 26·0%).

17β-*Pyrrolidin*-1′-*yl*-5α-*androstane methochloride* (IX)*. A mixture of 5α-androstan-17-one (5·0 g), formic acid (5 ml) and pyrrolidine (13·5 ml) was heated under reflux for 20 h (bath temperature 150–160°), then poured into 2N acetic acid. The filtered solution was basified, and the precipitate was filtered off, washed well with water and crystallized from methanol, giving colourless crystals of 17β-*pyrrolidin*-1′-*yl*-5α-*androstane* (3·5 g, 58%), m.p. 108–110°. (Found N, 4·1. $C_{23}H_{39}N$ requires N, 4·2%). Treatment with methyl iodide in methanol gave the *methiodide*, m.p. 276–278° (decomp.). (Found: C, 60·7; H, 9·1; I, 26·9. $C_{24}H_{42}IN$ requires C, 60·7; H, 9·1; I, 26·9%).

A solution of the methiodide (0·5 g) in water (250 ml) was stirred for 2 h at 100° with freshly precipitated silver chloride (from 0·25 g silver nitrate), and then filtered. The solid was washed with water and the combined filtrates were evaporated *in vacuo*. The residue was crystallized from 2N hydrochloric acid, giving 17β-*pyrrolidin*-1′-*yl*-5α-*androstane methochloride* (0·2 g, 49%), m.p. 273–274°. (Found: Cl, 9·6; N, 3·5. $C_{24}H_{42}ClN$ requires Cl, 9·4; N, 3·7%).

17α-*Pyrrolidin*-1′-*yl*-5α-*androstane methochloride* (X)*. A mixture of 17β-tosyloxy-5α-androstane (3·0 g) and pyrrolidine (45 ml) was heated in a sealed tube at 170–180° for 42 h. The excess of pyrrolidine was removed *in vacuo* and the residue was poured into dilute aqueous methanesulphonic acid. The acid solution was washed with ether and basified, and the precipitate was filtered off. Crystallization from acetone gave 17α-*pyrrolidin*-1′-*yl*-5α-*androstane* (0·7 g, 30%), m.p. 73–75°. (Found: C, 83·4; H, 11·8; N, 4·5. $C_{23}H_{39}N$ requires C, 83·9; N, 11·9; N, 4·2%). The *methiodide* had m.p. 263–265°. (Found: I, 26·8; N, 2·85. $C_{24}H_{42}IN$ requires I, 26·9; N, 3·0%).

A solution of the methiodide (0·6 g) in 50% aqueous methanol (10 ml) was run through a column of IR 400 ion-exchange resin (10 ml damp solid, chloride form). The column was washed with aqueous methanol (20 ml) and the combined eluates

* Experiment by Dr. D. Warburton.

were evaporated *in vacuo*. The residue, crystallized from methanol-ethyl acetate, gave the *methochloride* (0·4 g, 82·5%), m.p. 257–259° (decomp). (Found: Cl, 9·5; N, 3·3. $C_{24}H_{42}ClN$ requires Cl, 9·4; N, 3·7%).

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Some 5-hydroxytryptamine-like actions of fenfluramine: a comparison with (+)-amphetamine and diethylpropion

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The 5-hydroxytryptamine (5-HT)-like effects of fenfluramine have been investigated in mice in two different experiments. In a behavioural test in mice pretreated with tranylcypromine, fenfluramine caused stereotyped changes which were similar to those produced by 5-hydroxytryptophan (5-HTP) and were antagonized by methysergide or pretreatment with *p*-chlorophenylalanine (PCPA). Like 5-HTP, fenfluramine reduced the conditioned response in a one-trial conditioning test, an effect antagonized by methysergide or by PCPA pretreatment. The reduction in the conditioned response caused by a maximal electroconvulsion was also antagonized by PCPA, an effect prevented by 5-HTP. Equivalent anorectic doses of (+)-amphetamine and diethylpropion caused a small increase in stereotyped behaviour, but this was not modified by methysergide; both anorectic drugs were inactive in the one-trial conditioning test. It seems probable that the observed actions of fenfluramine are caused indirectly through the release of endogenous brain 5-HT.

Recently Jespersen & Scheel-Krüger (1970) reported that dogs treated with fenfluramine showed hypothermia and behavioural signs similar to those observed after an injection of 5-hydroxytryptophan (5-HTP) and that these effects were prevented by the 5-hydroxytryptamine (5-HT) antagonist, methysergide. These observations are consistent with earlier reports (Duhault & Verdavainne, 1967; Opitz, 1967) indicating that fenfluramine causes the release and subsequent depletion of brain 5-HT in rats. We have investigated the behavioural effects of fenfluramine in mice using two experimental situations to determine 5-HT-like activity. For comparison, two other anorectic agents, (+)-amphetamine and diethylpropion, were also examined.

METHODS

Behavioural effects

Groups of ten male mice (Tuck TFW strain), 18–24 g, were treated orally with 10 mg/kg of the monoamine oxidase inhibitor, tranylcypromine. Four h later the animals were given fenfluramine (8 or 32 mg/kg), (+)-amphetamine (2 or 8 mg/kg), diethylpropion (8 or 32 mg/kg), 5-HTP (10 or 40 mg/kg) or saline (10 ml/kg) by the intraperitoneal route. After 15 min, note was made of the number of animals which responded with head twitching, head waving, forelimb movement and backward locomotion, each feature being assigned an arbitrary score value of one. Further groups of mice were pretreated similarly with tranylcypromine but, in addition to the intraperitoneal dose of the anorectic agents, 5-HTP or saline, the animals received concomitantly a subcutaneous dose of the 5-HT-antagonist, methysergide, 20 mg/kg.

All the drug and dosage combinations were randomized (random permutations—Fisher & Yates, 1957) and the behavioural signs scored blindly. The mean score values were then calculated and compared for statistical significance using Student's *t*-test.

A further group of mice was given two oral doses of 200 mg/kg of the tryptophan hydroxylase inhibitor, *p*-chlorophenylalanine (PCPA) 48 and 24 h before tranylcypromine which was then followed 4 h later by an intraperitoneal dose of 32 mg/kg of fenfluramine.

One-trial conditioning test

Groups of ten male mice were placed individually into the well-lit side of a two-compartment box, which had a communicating hole to a larger dark area fitted with a grid floor. On entering the dark compartment, escape was prevented by a slide door, and the animals were given a conditioning foot-shock of 2 mA, 350 V for 5 s. When returned to the test situation 24 h later the time taken by the conditioned mice to enter the dark compartment (step-through latency, STL) was measured and compared with that of non-shocked (unconditioned) control mice. To evaluate the effects of drug treatment on conditioning, STL was measured in mice given intraperitoneal doses of fenfluramine (16 or 32 mg/kg), (+)-amphetamine (4 or 8 mg/kg), diethylpropion (16 or 32 mg/kg) or 5-HTP (20 or 40 mg/kg) 30 min before the foot-shock. Other groups of mice were pretreated similarly with intraperitoneal doses of fenfluramine or 5-HTP but, in addition, 10 or 20 mg/kg methysergide was given concomitantly subcutaneously.

In another experiment using groups of 16 mice, the effect of fenfluramine on STL was compared with that of a maximal electroconvulsion (MEC) induced through pinna electrodes (20 mA, 0·2 s, 50 Hz sinusoidal current) immediately after exposure to the foot-shock. In a further experiment, the effect of fenfluramine on STL was measured in animals given either saline or PCPA (200 mg/kg) by mouth 48, 24 and 2 h before the conditioning trial.

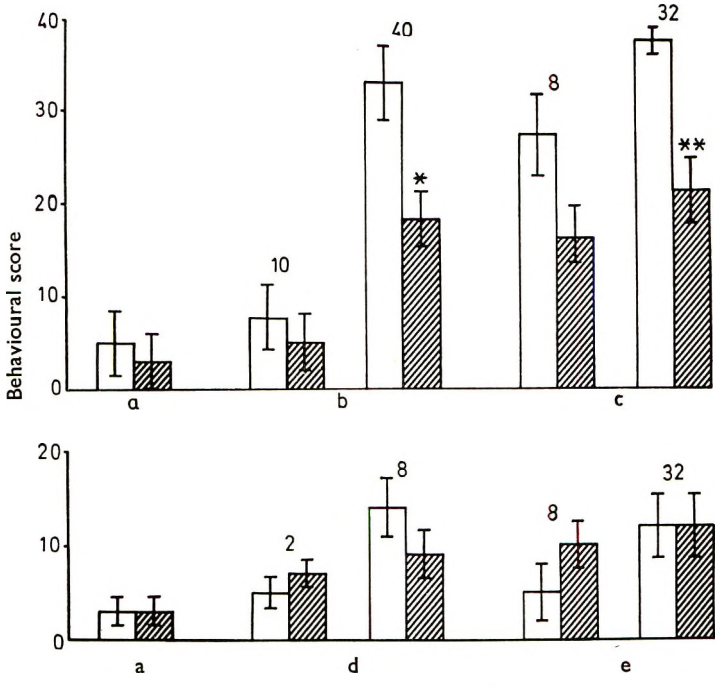
Tests for statistical significance of the differences between groups were made using the Mann-Whitney U-test (Siegel, 1956).

RESULTS

Behavioural effects

Control mice, pretreated with tranylcypromine, and injected with saline, showed only occasional head twitching, head waving and forelimb movement, with group scores of 3 to 5 compared with a possible maximum score of 40.

The effects of giving mice (pretreated with tranylcypromine to inhibit monoamine oxidase) 5-HTP, fenfluramine, diethylpropion or (+)-amphetamine are shown in Fig. 1. The low dose of 5-HTP (10 mg/kg) was without behavioural effect but 40 mg/kg caused a highly significant ($P < 0{\cdot}001$) increase in the behavioural score to 32. Fenfluramine produced the same behavioural syndrome as the high dose of 5-HTP: 8 and 32 mg/kg of fenfluramine gave scores of 27 and 37 which were significantly different ($P = 0{\cdot}01$–$0{\cdot}001$ and $P < 0{\cdot}001$ respectively) from that of the saline-treated control animals. Methysergide (20 mg/kg) caused no change in the behaviour of the control animals but gave a significant reduction (43%, $P = 0{\cdot}02$–$0{\cdot}01$ and 44%, $P < 0{\cdot}001$) in the behavioural scores elicited by both 5-HTP (40 mg/kg) and



FIG, 1. Behavioural effects of a, saline, b, 5-HTP, c, fenfluramine, d, (+)-amphetamine, and e, diethylpropion in tranylcypromine-pretreated mice. Open columns represent behavioural scores produced by intraperitoneal doses (mg/kg) of the drugs alone; hatched columns show the effects of the drugs administered concomitantly with methysergide, 20 mg/kg subcutaneously. Bars give the standard error of the mean behavioural scores. Asterisks indicate a statistically significant antagonism by methysergide (Student's *t*-test, $*P = 0{\cdot}02$–$0{\cdot}01$, $**P < 0{\cdot}001$).

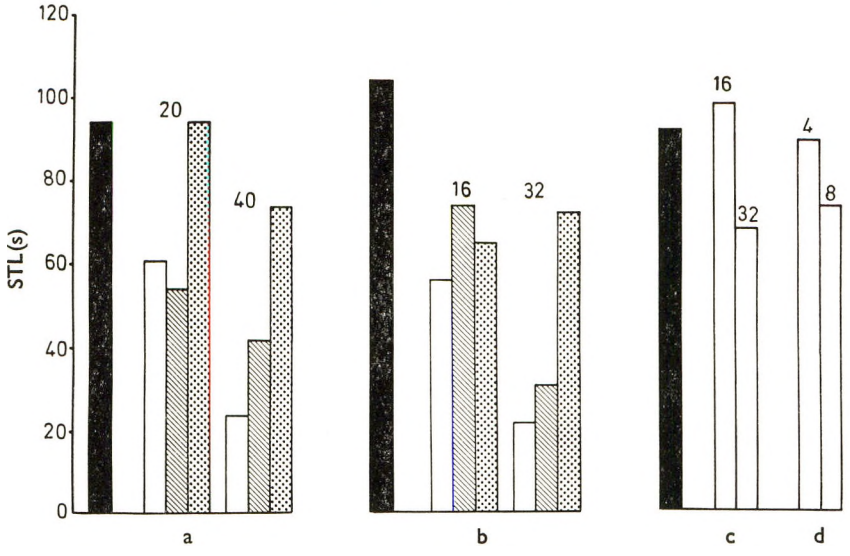
fenfluramine (32 mg/kg). Pretreatment with two oral doses of 200 mg/kg of PCPA caused a significant reduction ($P < 0{\cdot}001$) of 64% in the behavioural score elicited by the high dose of 32 mg/kg of fenfluramine.

The highest doses of (+)-amphetamine (8 mg/kg) or diethylpropion (32 mg/kg) also caused a significant increase in behavioural responses ($P = 0{\cdot}01$–$0{\cdot}001$ and $P = 0{\cdot}05$–$0{\cdot}02$ respectively), but both drugs were much less active than fenfluramine and 5-HTP, with maximum scores of only 14 and 12. Methysergide (20 mg/kg) failed to modify the behavioural effects caused by (+)-amphetamine or diethylpropion.

Single trial conditioning experiments

In eight experiments, the mean step-through latency of unconditioned (non-shocked) saline treated mice was 12·8 s. After a conditioning foot-shock the STL was prolonged, the mean values ranging from 77 to 104 s (Figs 2 and 3).

5-HTP gave a dose-related reduction in STL (Fig. 2). With the highest dose of 5-HTP (40 mg/kg) the reduction in STL was highly significant ($U = 0$, $P < 0{\cdot}001$) the mean value of 24 s approaching that of the unconditioned control animals. Methysergide (10 and 20 mg/kg) antagonized the reduction in STL caused by 5-HTP but this effect was statistically significant ($U = 15$, $P = 0{\cdot}01$–$0{\cdot}001$) only for the high dose of methysergide given with 40 mg/kg of 5-HTP.



FIG, 2. The effect on step-through latency of intraperitoneal doses of a, 5-HTP, b, fenfluramine, c, diethylpropion and d, (+)-amphetamine administered alone or concomitantly with methysergide given subcutaneously. Solid columns indicate the results obtained in control saline-treated animals; the other columns show the effects of the drugs alone (open columns) and combined with methysergide, 10 mg/kg (hatched columns) or 20 mg/kg (stippled columns).

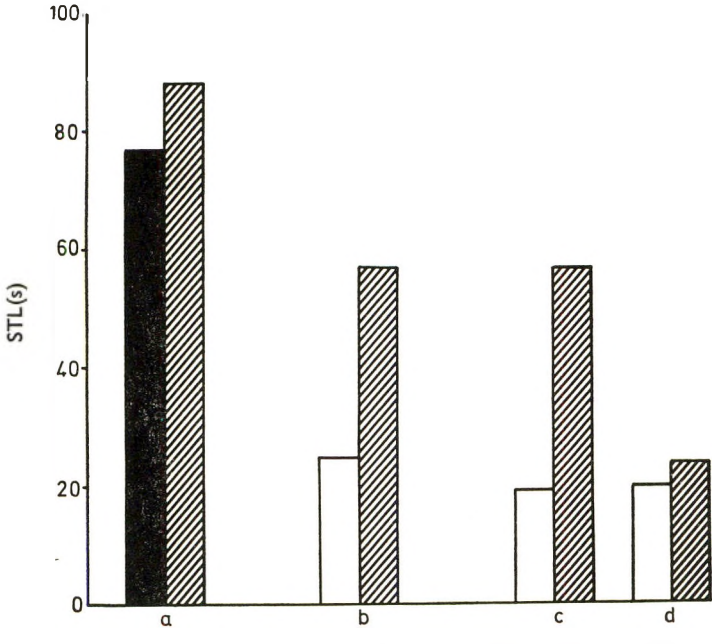


FIG. 3. The effects of oral pretreatment with *p*-chlorophenylalanine (PCPA), 3 × 200 mg/kg, on the reduction of step-through latency caused by a, saline, b, fenfluramine (32 mg/kg), c, maximal electro-convulsion (MEC) or d, MEC plus prior treatment with 5-HTP (20 mg/kg). Solid column indicates the results obtained in control saline-treated animals; open columns show the effects of the treatments alone and hatched columns the effects of the treatments after PCPA.

Fenfluramine, like 5-HTP, also gave a dose-related reduction in STL (Fig. 2) which was statistically significant ($U = 1$, $P < 0{\cdot}001$) only with the high dose of 32 mg/kg, which reduced the mean STL to 22 s. Methysergide (20 and 40 mg/kg) antagonized this reduction in STL caused by fenfluramine and this antagonism was again statistically significant ($U = 21$, $P = 0{\cdot}05$–$0{\cdot}025$) only with the high dose combination of the two drugs. Pretreatment with three oral doses of 200 mg/kg of PCPA also significantly antagonized ($U = 24$, $P = 0{\cdot}05$–$0{\cdot}025$) the reduction in STL caused by 32 mg/kg of fenfluramine (Fig. 3).

In contrast to 5-HTP and fenfluramine, diethylpropion (16 and 32 mg/kg) and (+)-amphetamine (4 and 8 mg/kg) did not produce a statistically significant reduction in STL (Fig. 2).

Like 5-HTP and fenfluramine, a maximal electroconvulsion (MEC) elicited immediately after conditioning, also reduced STL to the level found in unconditioned control animals (Fig. 3). Pretreatment with PCPA significantly antagonized MEC ($U = 19{\cdot}5$, $P < 0{\cdot}001$), an effect prevented by the prior intraperitoneal administration of 20 mg/kg of 5-HTP ($U = 20{\cdot}5$, $P < 0{\cdot}001$).

DISCUSSION

Experiments with tranylcypromine-pretreated mice showed that a high dose of the 5-HT precursor, 5-HTP, caused characteristic stereotyped behaviour which was antagonized by the 5-HT blocking agent, methysergide. In agreement with the work of Jespersen & Scheel-Krüger (1970) in dogs, we also found that fenfluramine caused 5-HTP-like behavioural changes which, similarly, were blocked by methysergide and, moreover, were prevented by the prior administration of the tryptophan hydroxylase inhibitor PCPA (Koe & Weissman, 1966; Jéquier, Lorenburg & Sjoerdsma, 1967). In contrast to these findings, although diethylpropion and (+)-amphetamine in doses equianorectic with fenfluramine caused a significant change in behaviour, their effect was small and not modified by methysergide, indicating that they are unlikely to be exerting a major action on 5-HT mechanisms.

In the one-trial conditioning experiments, STL was prolonged 24 h after a conditioning foot-shock, and this prolongation was antagonized by MEC elicited immediately after conditioning. This result confirms the work of Essman (1968) who showed that consolidation of memory could be disrupted by an electroconvulsion, an effect which was associated with an increase in the brain concentrations of 5-HT. Our findings are consistent with the conclusion that prolongation of STL can be modified by an action on 5-HT mechanisms, as the prior administration of 5-HTP prevented the increase in STL following conditioning and this action was antagonized by methysergide. Moreover, our experiments have also shown that reduction of STL by MEC is antagonized by prior treatment with PCPA and this effect, in turn, is prevented by the concomitant administration of 5-HTP.

In agreement with the results of the behavioural experiments, the effect of fenfluramine in the one-trial conditioning tests mimicked that of 5-HTP and also that of MEC. The reduction in STL caused by fenfluramine was antagonized by methysergide and also by pretreatment with PCPA. In the doses used, neither (+)-amphetamine nor diethylpropion significantly affected STL, indicating a difference in the actions of these anorectic drugs.

The effects of fenfluramine on behaviour and on STL, their similarity with the effects of 5-HTP and their modification by both methysergide and PCPA can all be

explained by an increase in the activity of a 5-HT-sensitive system. Although brain concentrations of 5-HT were not measured in our experiments, it seems probable that the action of fenfluramine may be exerted indirectly through the release of endogenous brain 5-HT. Duhault & Verdavainne (1967) showed that fenfluramine in oral doses of 10 to 30 mg/kg caused depletion of brain 5-HT and 5-hydroxyindoleacetic acid, maximal reductions appearing approximately 4 h after dosing. In our experiments, observations were made 30 min and 1 h after intraperitoneal and oral treatment respectively, when elevated brain concentrations of 5-HT could be anticipated.

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Effect of amphetamine on the uptake, release and effectiveness of xylocholine in the guinea-pig vas deferens

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Amphetamine sulphate will both reverse and prevent the adrenergic neuron blocking action of xylocholine (TM10 bromide) on the response of the guinea-pig isolated vas deferens to transmural electrical stimulation. A concentration of amphetamine sulphate capable of reversing the effect of xylocholine does not produce a significant reduction in the tissue concentration of ^{14}C-TM10 iodide in the vas deferens. Although amphetamine reduces the rate of uptake of xylocholine, it does not prevent uptake. Comparisons of tissue concentrations with the degree of blockade produced in the normal and the amphetamine-treated vas deferens suggest that if the actions of amphetamine are to be accounted for entirely by displacement of xylocholine or by changes in uptake of xylocholine, only a very small percentage of the total tissue content of xylocholine can be involved in the production of its effects.

It is well established that the adrenergic neuron blocking action of a variety of compounds can be prevented by the prior administration of amphetamine and that this agent will also reverse an established adrenergic neuron blockade (Wilson & Long, 1960; Day, 1962; Follenfant & Robson, 1970). Tissue concentrations of *NNN*-trimethyl-2-(2,6-xylyloxy)ethylammonium iodide (TM10 iodide) achieved during the establishment of adrenergic neuron blockade in a tissue receiving mainly adrenergic innervation have now been measured to see if these tissue concentrations were modified by the administration of amphetamine in doses known to be effective in reversing or preventing adrenergic neuron blockade.

METHODS

NN-*dimethyl*-N-*methyl*-[^{14}*C*]-2-(2,6-*xylyloxy*)*ethylammonium iodide* (*^{14}C-TM*10 *iodide*). Methyl[^{14}C]iodide (0·5 mCi, 0·009 mmol) was condensed in a vacuum onto *NN*-dimethyl-2-(2,6-xylyloxy)ethylamine (0·42 mmol) in dry acetone (5 ml) and the mixture was allowed to stand at room temperature for 24 h. Methyl iodide (0·8 mmol) was then condensed onto the reaction mixture which was allowed to stand at room temperature (20°) for a further 24 h. The acetone and excess methyl iodide were removed in a vacuum and a white crystalline deposit of ^{14}C-TM10 iodide remained (0·42 mmol: m.p. 226–228°: specific activity, 1·31 mCi/mmol). [A sample of this material prepared under the same conditions but omitting the addition of methyl [^{14}C] iodide had a m.p. of 229–230°. Found: C, 46·35; H, 6·4; N, 4·4%. $C_{13}H_{22}INO$ requires C, 46·6; H, 6·61; N, 4·17%.]

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Thin-layer chromatography of the ^{14}C-TM10 iodide followed by scanning of the plates in a Tracerlab Scanner and by autoradiography showed a single spot in the t.l.c. systems in Table 1.

Preparation of tissues. Vasa deferentia were removed from freshly-killed guinea-pigs (weight, 300–600 g) and transferred to Krebs solution (NaCl, 6·9; KCl, 0·35; $CaCl_2.6H_2O$, 0·65; $MgSO_4.7H_2O$, 0·28; K_2HPO_4, 0·16; $NaHCO_3$, 2·1; glucose, 2·0 g/litre: gassed with 5% carbon dioxide in oxygen) at room temperature.

Transmural stimulation. Vasa deferentia were suspended in Krebs solution at 37° and were allowed to equilibrate for 0·25 h. Electrical stimulation (rectangular wave pulses, 50/s, 0·5 ms duration, supramaximal voltage) was applied for 7 s every 0·25 h (or as required) through transmural electrodes (Birmingham & Wilson, 1963) and contractions were recorded on a Heathkit chart recorder via an isotonic transducer. Two or three control responses were elicited from the tissue before any drugs were added to the bath and all responses were expressed as a percentage of these control responses. Multiple determinations of the effect of the several treatments employed were made on different vasa deferentia and means and standard errors of the size of the response to transmural stimulation at each application were calculated.

Table 1. *Thin-layer chromatography systems used and the approximate R_F values of the single spots obtained on chromatography of ^{14}C-TM10 iodide.*

Plate	Solvent	R_F
0·25 mm silica gel G (Stahl)	Dry acetone–water (20:30) containing 1% w/total volume NaBr	0·55
"	Ethanol–water (50:2) containing 1% w/total volume NaBr	0·3
0·25 alumina for t.l.c. (Hopkin & Williams)	Cyclohexane–chloroform–glacial acetic acid–isopropanol (32:32:4:6)	0·3
"	Cyclohexane–chloroform–glacial acetic acid–ethanol (32:32:8:8)	0·55
"	Cyclohexane–chloroform–glacial acetic acid (45:45:11)	0·45

Uptake of TM10 iodide. Vasa deferentia were incubated at 37° in Krebs solution gassed with water-saturated 5% carbon dioxide in oxygen and containing 1·91 μg/ml ^{14}C-TM10 iodide with or without the presence of 10 μg/ml amphetamine sulphate. On completion of the incubation (which was for up to 1·5 h) the tissues were removed from the solution, blotted dry on filter paper, weighed on a torsion balance, placed on small (≈150 mg) squares of filter paper and allowed to air-dry for 1–3 h. The tissue [^{14}C] content was determined by burning the tissue and filter paper in a Packard Sample Oxidizer (model 305) and counting the radioactivity of the resulting solutions in a Packard Liquid Scintillation Spectrometer (model 3320). Results, corrected for counting efficiency (using external standardization), are expressed as ng ^{14}C-TM10 iodide per g wet weight tissue (mean and standard error). Results have not been corrected for recovery of ^{14}C from the combustion process which was 84·9 ± 0·5% as determined by combustion of known amounts of ^{14}C-TM10 iodide spotted on to Kleenex tissue.

Release of ^{14}C-TM10 iodide. Tissues were incubated with 1·91 μg/ml ^{14}C-TM10 iodide for 1·5 h as described above and were then removed from the radioactive

solution, washed for 3 s in Krebs solution at 37° and placed in tissue baths at 37° containing either drug-free Krebs solution or Krebs solution plus (±)-amphetamine sulphate (10 μg/ml). The tissue baths were drained and refilled with fresh solutions every 0·25 h and tissue [^{14}C] content was determined at suitable intervals as described above.

The following drugs were used: (±)-amphetamine sulphate, cinchocaine hydrochloride, *NN*-dimethy-*N*-methyl-[^{14}C]-2-(2,6-xylyloxy)ethylammonium iodide ^{14}C–TM10 iodide and *NNN*-trimethyl-2-(2,6-xylyloxy)ethylammonium bromide (TM10 bromide: xylocholine) and all concentrations are expressed in terms of these salts.

Tests for significance of difference were performed using Students *t*-test.

RESULTS

Transmural stimulation. Transmural stimulation of vasa deferentia as described and in the absence of any drugs, elicited reproducible responses from the tissue for at least 6 h (5 exp). In the presence of 1·64 μg/ml of TM10 bromide (xylocholine) (equivalent to 1·91 μg/ml ^{14}C-TM10 iodide) a blockade of the effects of transmural stimulation was produced which became progressively greater over the 1·5 h during which the drug was allowed to remain in contact with the tissue (Fig. 1). The response elicited by transmural stimulation 1·5 h after the addition of the drug to the tissue bath was 28·2 ± 6·6% of the control response. Immediately after this response had been obtained, the bath was drained and refilled with fresh drug-free Krebs solution and this was repeated every 0·25 h for 1 h during which time the

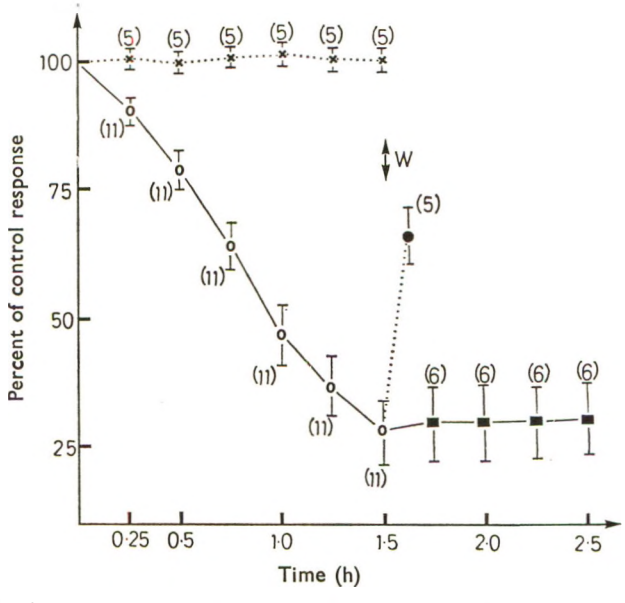

FIG. 1. Showing the means and standard errors of the responses (percentage of control response) of several (figures in parentheses) guinea-pig vasa deferentia to transmural stimulation (rectangular wave pulses, 50/s, 0·5 ms duration, supramaximal voltage: 7 s every 0·25 h) plotted against the time (h) the tissue was exposed to the following treatments: × . . . ×, TM10 bromide (1·64 μg/ml) and amphetamine sulphate (10 μg/ml) administered simultaneously. ○——○ TM10 bromide (1·64 μg/ml) alone. At W, the tissue bath was drained and refilled with fresh Krebs solution (■——■) or with Krebs solution plus 10 μg/ml amphetamine sulphate (○ . . . ●) and this process was then repeated every 0·25 h. In the latter case transmural stimulation was applied 7 min after addition of the amphetamine sulphate.

tissue was stimulated transmurally every 0·25 h as usual. Little reversal of the blockade was produced in this 1 h period; the response was not significantly different ($P > 0{\cdot}9$) from that obtained before the washing process started (Fig. 1).

The addition of amphetamine sulphate to the tissue bath immediately after the first wash did produce a significant reversal of the blockade ($P < 0{\cdot}01$). The response to transmural stimulation 7 min after the addition of amphetamine being 65·9 ± 5·0% of the control response. This reversal of the blocking action of TM10 bromide by amphetamine was fast in onset and in each of 5 additional experiments a significant reversal of the blockade was seen on application of transmural stimulation 30 s after the addition of amphetamine.

When 1·64 μg/ml TM10 bromide and 10 μg/ml amphetamine sulphate were administered to the tissue simultaneously at the start of the experiment, no blockade of the response to transmural stimulation developed within 1·5 h, after which time the experiment was terminated (Fig. 1). At this time the response to transmural stimulation was 100·8 ± 0·6% of the control response and was not significantly different ($P > 0{\cdot}3$) from the response of the tissue at this time in the absence of all drugs.

The blockade of the response to transmural stimulation produced by 1·5 μg/ml cinchocaine hydrochloride was not prevented (2 exps) or reversed (3 exps) by the administration of 10 μg/ml of amphetamine sulphate.

Tissue concentrations of ^{14}*C-TM*10 *iodide.* On incubation of vasa deferentia with 1·91 μg/ml ^{14}C-TM10 iodide (equivalent to 1·64 μg/ml TM10 bromide) there was a fast initial uptake of ^{14}C-TM10 iodide during the first 0·25 h of the incubation and thereafter tissue concentrations continued to rise for at least 1·5 h (Table 2 and Fig. 2). Immediately after this time, tissues were removed from the radioactive solution and transferred to tissue baths containing drug-free Krebs solution or Krebs solution containing 10 μg/ml of amphetamine sulphate. After 7 min further incubation in these solutions, the tissue content of the two groups of vas deferens were 8104 ± 594 and 7963 ± 369 ng ^{14}C-TM10 iodide/g tissue respectively, and were not significantly

Table 2. *Tissue concentrations of* ^{14}*C-TM*10 *iodide* (ng/g) *in guinea-pig vasa deferentia* (*means* ± *standard errors*) *during incubation in Krebs solution at* 37° *containing* 1·91 μg/ml ^{14}*C-TM*10 *iodide with or without* 10 μg/ml *amphetamine sulphate.* The figures in parentheses indicate the number of vasa deferentia used for each determination. Levels of significance were calculated using Students *t*-test.

Duration of incubation (h)	^{14}C-TM10 iodide ng/g tissue	
	Without amphetamine	With amphetamine
0·25	3033·3 ± 402·9 (6)	1338·3 ± 60·0** (6)
0·5	4570·8 ± 433·7 (8)	1967·3 ± 121·3*** (6)
1·0	5931·2 ± 513·6 (8)	3044·5 ± 344·1** (6)
1·5	8870·8 ± 582·5 (10)	3941·2 487·9*** (6)

** = $P \leq 0{\cdot}01$.
*** = $P \leq 0{\cdot}001$.

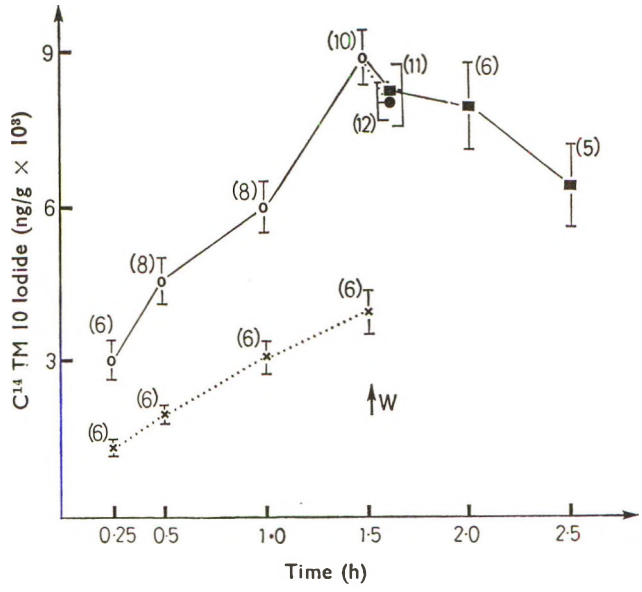

FIG. 2. Means and standard errors of the tissue concentrations (ng/g) of ^{14}C-TM10 iodide attained in guinea-pig vas deferens incubated at 37° in Krebs solution containing 1·91 μg/ml ^{14}C-TM10 iodide alone (○——○) or plus 10 μg/ml amphetamine sulphate (× . . . ×). At W, the tissues incubated in the absence of amphetamine were transferred to a tissue bath containing fresh Krebs solution (■——■) or Krebs solution containing 10 μg/ml amphetamine sulphate (. . . ●). The figures in parentheses indicate the number of tissue estimations contributing to each point.

different ($P > 0{\cdot}3$). Some vasa deferentia were allowed to remain in drug-free Krebs solution for up to 1 h during which time no marked fall in the tissue content of ^{14}C-TM10 iodide was observed.

Incubation of vasa deferentia with 1·19 μg/ml ^{14}C-TM10 iodide in the presence of 10 μg/ml of amphetamine sulphate also produced a steady increase in the tissue content of ^{14}C-TM10 iodide over 1·5 h (Table 2). The tissue concentrations attained however were only $\approx$30–50% of those obtained during incubation with ^{14}C-TM10 iodide alone at each incubation time investigated (Table 2).

DISCUSSION

The results show that the slowly-developing blockade produced by TM10 bromide (xylocholine) of the response of the guinea-pig vas deferens to transmural stimulation can be completely prevented by the simultaneous administration of amphetamine sulphate (Fig. 1).* Determinations of the tissue content of vasa deferentia incubated with an equivalent concentration of ^{14}C-TM10 iodide show that there is a slow accumulation of ^{14}C-TM10 iodide in the tissue and that this is not prevented by amphetamine sulphate. Amphetamine does however reduce the rate of accumulation and the magnitude of the tissue concentrations attained in a given time (Fig. 2). After 1·5 h incubation in its presence tissue concentrations were approximately equal to those attained after 22 min incubation with ^{14}C-TM10 iodide alone. At

* This is unlikely to be due to any "direct" action of amphetamine on the tissue since the actions of cinchocaine hydrochloride were not modified by amphetamine.

this time, in the absence of amphetamine, the response of the tissue to transmural stimulation was reduced to about 85% of the control response but in its presence no corresponding blockade was seen. These results suggest that though the reduction in uptake of ^{14}C-TM10 iodide produced by amphetamine may contribute to a reduction in the effects of TM10 bromide, this effect of amphetamine is insufficient in itself to account completely for the reduction in TM10 bromide effects.

When vasa deferentia that had been treated with TM10 bromide for 1·5 h were then treated with amphetamine, a marked reversal of the blockade of the response to transmural stimulation was produced. This treatment did not however produce any significant change in the total tissue content of ^{14}C-TM10 iodide. Amphetamine cannot therefore displace from the tissue much of the total tissue content of ^{14}C-TM10 iodide. Since the action of amphetamine under these conditions can be demonstrated as soon as 30 s after administration, it seems unlikely that a relocation of any major portion of the ^{14}C-TM10 within the tissue could take place within the short time period.

It seems likely therefore that neither prevention of uptake nor displacement of TM10 can entirely account for the ability of amphetamine to prevent or to reverse the blocking action of TM10 in the guinea-pig vas deferens unless only a very small proportion of the total tissue content of xylocholine is involved in the production of its actions.

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The structure of buckwheat, *Fagopyrum esculentum* Moench

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The morphology and anatomy of the aerial parts of *Fagopyrum esculentum* have been described and the most important microscopical characters of value in the identification of commercial Buckwheat have been indicated.

Large quantities of buckwheat are cultivated throughout Europe and North America for the production of rutin. The species usually employed is *Fagopyrum esculentum*, a fast-growing annual from which a crop can be collected within eight to ten weeks of sowing. As the dried commercial material, consisting of the flowering shoots, is usually in a much-broken condition, the histology of the leaves, stems and inflorescence have been examined with a view to finding the diagnostic characters for the identification of this material.

The plant was first described by Moench (1794) and the detailed structure of the seeds was studied by Parkinson (1934). The surface characters of the leaves have been illustrated by Boratyńska (1959) and Miège (1910) investigated the source of the rutin in the stem and leaves but did not describe details of the anatomical structure.

MATERIALS

Plants were grown in 1966 and 1967 from two different commercial samples of seed, and harvested when in flower. Also, a sample was collected from a growing crop in Northern Italy in July, 1969. All the plants used complied with the type description for the species (Hector, 1938).

LEAVES

Macroscopical characters (Fig. 1·1)

Leaves measure from 3 to 11 cm in length and 1·5 to 10 cm in breadth; those on the upper part of the plant are cordate to sagittate and the larger, lower leaves are cordate to ovate. The margin is entire or slightly sinuous; the apex is acuminate. The leaves are bright green and thin, with the upper darker than the lower surface. The surfaces are finely pubescent, especially over the veins, which are more prominent on the lower surface.

The venation is palmate, with a central vein (midrib) running to the apex and three main lateral veins on each side, all arising at the same point at the base of the lamina; these lateral veins anastomose about 2 to 3 mm from the margin. The subsidiary venation is reticulate. Most of the leaves are petiolate although a few on the upper part of the stem are sessile. The petiole, 0·5 to 8 cm in length and 0·5 to 2 mm in diameter, is cylindrical with a single longitudinal groove on the adaxial surface, along the two edges of which occur a row of papillae. There is an ochreate stipule at the base of each leaf (Fig. 3·8).

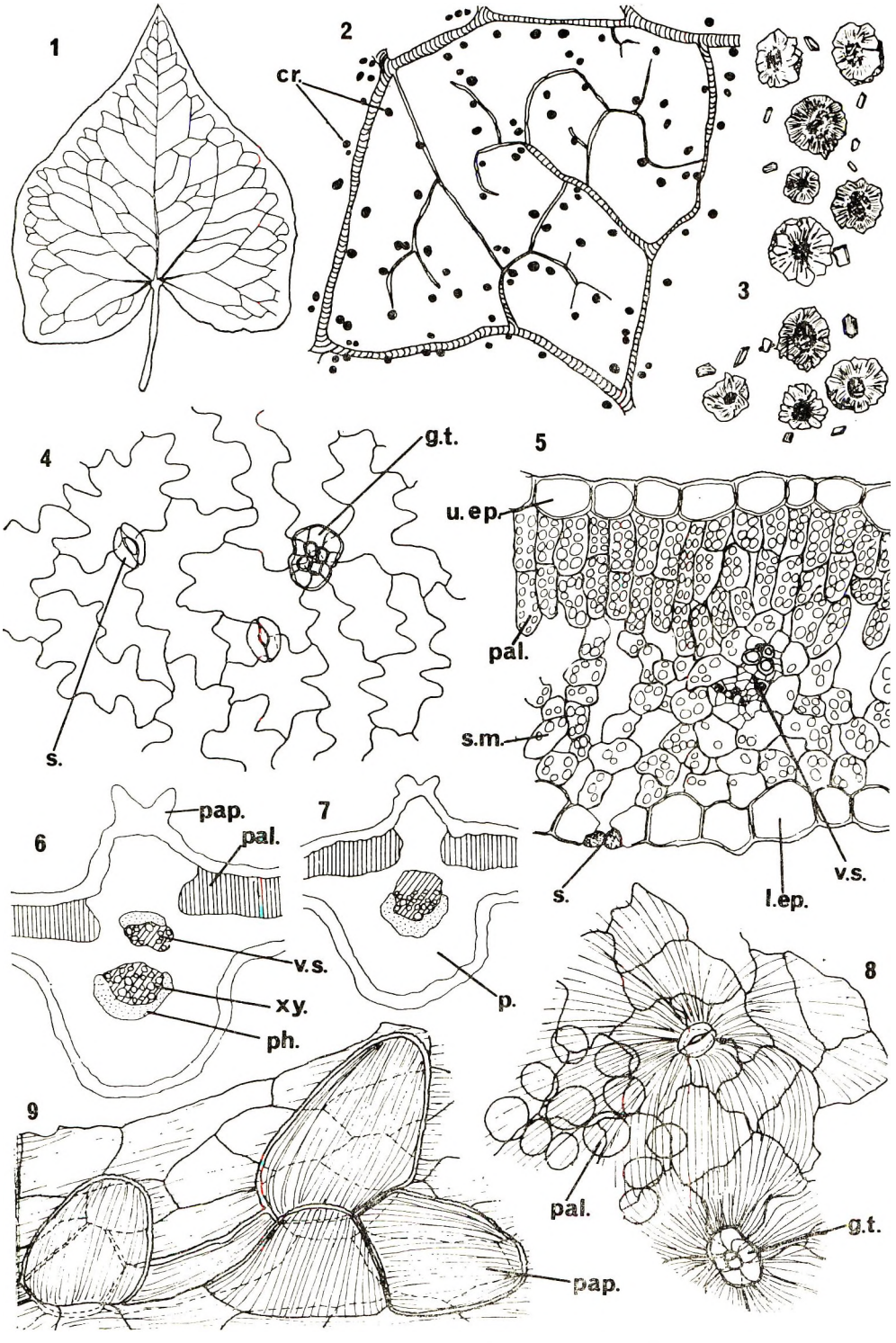

FIG. 1. *Fagopyrum esculentum*, leaf. 1, whole leaf × ½, showing the vein arrangement; 2, lower surface showing distribution of calcium oxalate; 3, calcium oxalate crystals; 4, lower epidermis in surface view; 5, transverse section of the lamina; 6, transverse section of the midrib near the petiole; 7, transverse section of the midrib near the apex; 8, upper epidermis in surface view; 9, upper epidermis over a vein in surface view showing papillae. 2, 6, 7 × 45; 3–5, 8, 9 × 220. cr = calcium oxalate crystals; g.t. = glandular trichome; l.ep. = lower epidermis; pal. = palisade; pap. = papilla; ph. = phloem; s. = stoma; s.m. = spongy mesophyll; u.ep. = upper epidermis; v.s. = vascular strand; xy. = xylem.

Histological characters

Lamina. The upper epidermis (Fig. 1·8) consists of a layer of thin-walled cells, polygonal to elongated in surface view with slightly sinuous anticlinal walls; the cuticle is strongly striated. Anomocytic and occasional anisocytic stomata are present. The scattered glandular trichomes have a stalk composed of four contiguous cells and a multicellular, sub-spherical to ovoid head; numerous short, bluntly-tapering papillae covered by a strongly striated cuticle, occur over the veins (Fig. 1·9). The mesophyll is clearly differentiated. The palisade consists of two (or occasionally three) layers of cells in which the length varies from one to three times the breadth; the cells contain numerous chloroplasts. The spongy mesophyll is loosely packed, with numerous intercellular spaces; the cells are irregular and contain scattered chloroplasts. Occasional cells contain cluster crystals of calcium oxalate up to about 45 μm in diameter or, less frequently, prisms about 12–15 μm long (Fig. 1·2, 1·3).

The cells of the lower epidermis (Fig. 1·4) are irregular and slightly elongated in surface view with thin, very sinuous anticlinal walls; the outer periclinal walls are slightly convex and covered with a thin cuticle which is not striated. Anomocytic and anisocytic stomata occur more frequently than on the upper surface but are not present over the veins. There are more glandular trichomes than on the upper epidermis and they are similar in structure. Papillae occur only rarely over the veins on the lower surface.

Midrib. In the region of the midrib the cells of the upper epidermis are larger than those of the interneural lamina and two adjacent cells immediately above the centre of the meristele are much enlarged and extended to form two rows of papillae (Fig. 2·1). The outer periclinal walls are covered, as on the lamina, by a striated cuticle which also covers the papillae. The cells are longitudinally elongated with nearly straight anticlinal walls; the cuticle is not striated. Immediately inside the upper epidermis, in the region of the papillae, there is a group of parenchymatous cells with slightly thickened walls. The remainder of the area between the upper and lower epidermises and the meristele is filled with thin-walled parenchymatous cells, some of which contain cluster crystals or, occasionally, prisms of calcium oxalate.

The structure of the meristele, as seen in a transverse section, varies with the position along the length of the leaf. At the base a small, additional group of vascular tissue occurs between the main central group and the upper epidermis (Fig. 1·6). This second vascular bundle continues up the leaf to a point approximately half way between the base and the apex (Fig. 1·7). In the main bundle the xylem forms an irregular to semicircular mass with the phloem occurring as a fairly wide band on the lower side. Where the second bundle is present it is orientated with the xylem forming an irregular compact mass on the inside and phloem towards the outside; thin-walled parenchymatous tissue separates the two groups of xylem.

The xylem is composed of vessels with thick, lignified walls, interspersed with small, thin-walled unlignified xylem parenchymatous cells. In transverse section the phloem appears as small, thin-walled cells, some of which contain dense contents. (Fig. 2·1).

Petiole. The epidermis consists of thin-walled polygonal cells, elongated in surface view with a strongly striated cuticle. Stomata are present and the subsidiary cells are much smaller than the remainder of the epidermal cells and are approximately

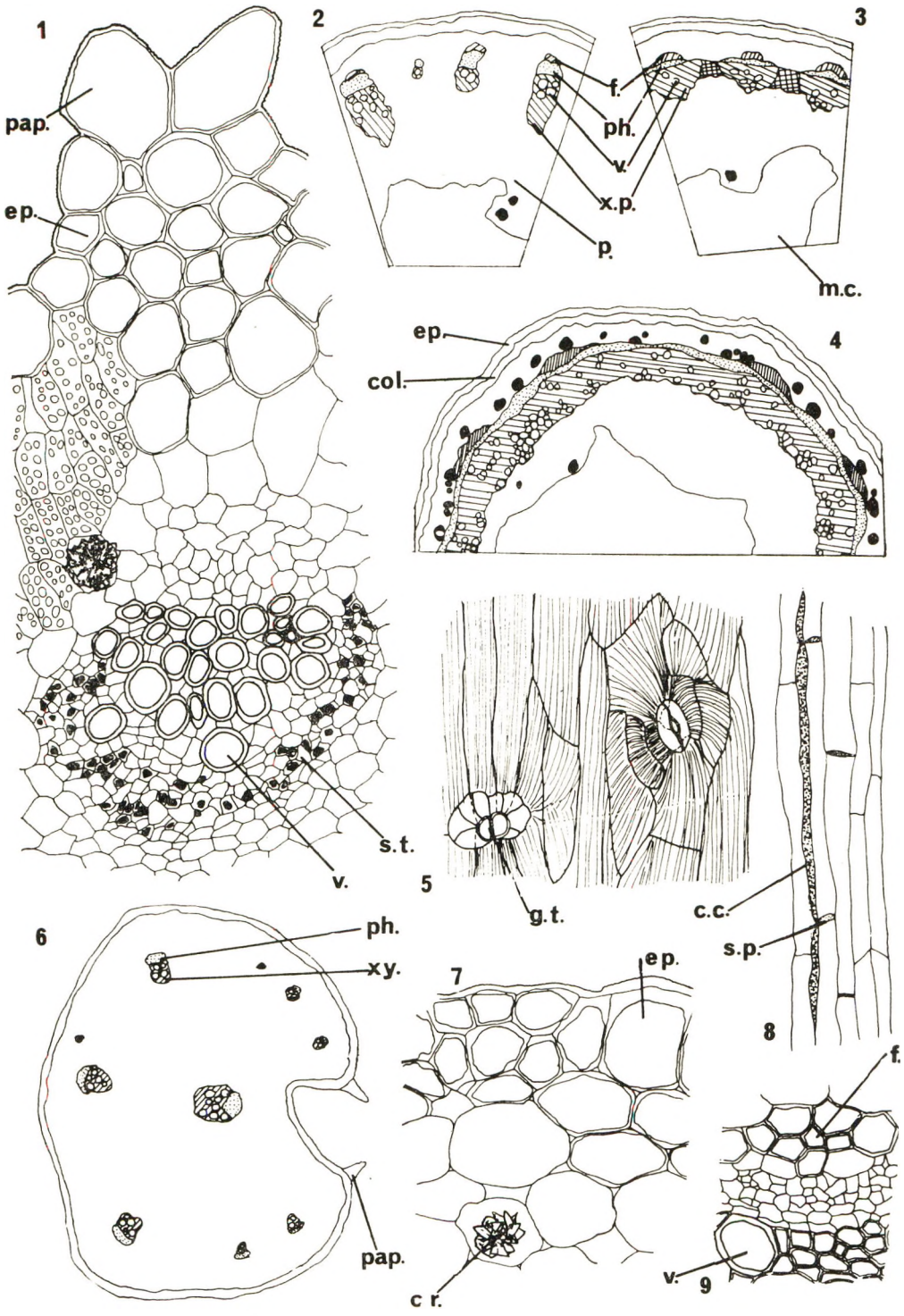

FIG. 2. *Fagopyrum esculentum*, leaf and stem. 1, transverse section of the midrib near the apex; 2, 3, 4, diagrams of transverse sections of young, older and fully mature stems respectively; 5, epidermis of the petiole in surface view; 6, diagram of a transverse section of the petiole; 7, transverse section of outer tissues of a young stem; 8, sieve tissue in longitudinal section; 9, transverse section through the vascular tissues of an older stem. 2, 3, 4 and 6 × 45, others × 220. c.c. = companion cell; col. = collenchyma; cr. = calcium oxalate crystal; ep. = epidermis; f. = fibres; g.t. = glandular trichome; m.c. = pith cavity; p. = parenchyma; pap. = papilla; ph. = phloem; s.p. = sieve plate; s.t. = sieve tube; v. = vessel; x.p. = xylem parenchyma; xy. = xylem.

radially arranged (Fig. 2·5). There are scattered glandular trichomes similar to those on the leaves, and along each edge of the longitudinal furrow there is a single row of elongated papillae.

A transverse section through the petiole is sub-reniform in outline with the groove forming a well-marked indentation, on either side of which occur the papillae (Fig. 2·6). The ground tissue is composed of thin-walled parenchyma with occasional cells containing cluster crystals of calcium oxalate. The main vascular bundle occurs near the central region in line with the groove on the upper surface; it is composed of an irregular mass of xylem with a compact mass of phloem towards the outside. Other, similarly-constructed but slightly smaller, vascular bundles occur round the periphery; usually three such bundles are found in the lower part of the petiole and four much smaller bundles, composed of a few elements only, on each side of the groove (Fig. 2·6). The structure of the xylem and phloem in the bundles is similar to that of the meristele of the midrib except that a cambium is present in the larger bundles.

STEM

Macroscopical characters

The stems are cylindrical with a central hollow and measure up to 1 cm or more in diameter at the base. They are pale green or reddish and are glabrous except at the nodes, where they are finely pubescent.

Histological characters

The epidermal cells have slightly thickened outer periclinal walls and are covered by a thin, finely striated cuticle; in surface view they are axially elongated. Stomata, similar to those found on the petiole, are present but they are not abundant (Fig. 3·9). Glandular trichomes, similar to those found on the stipule, and papillae occur at the nodes, but the internodal regions are glabrous. Beneath the epidermis are one or two layers of collenchymatous cells and the remainder of the cortex is composed of thin-walled parenchymatous cells, some of which contain cluster crystals of calcium oxalate (Fig. 2·7).

The vascular tissue in a young stem is arranged in a number of discrete bundles (Fig. 2·2) but in a more mature stem it forms a complete cylinder when seen in transverse section (Fig. 2·3, 2·4). The medullary rays, which are unlignified in the young stem, become lignified in the xylem region of a more mature stem and when the stem is fully mature the medullary rays are frequently difficult to discern.

In a young stem a group of fibres occurs outside each of the larger vascular bundles and as the stem matures additional fibres are developed but they do not form a continuous pericyclic layer. Individual fibres have moderately thickened, lignified walls with numerous pits and reach a length of about 1 to 1·5 cm (Figs 2·9, 3·12).

The secondary phloem is composed of groups of sieve tubes and phloem parenchyma traversed by fairly wide medullary rays. The sieve tubes are narrow with transverse or slightly oblique sieve plates and they are usually accompanied by small companion cells with dense contents. The phloem parenchymatous cells are also small and narrow and are very thin-walled (Fig. 2·8).

The cambiform tissue consists of two or three layers of thin-walled, tangentially elongated cells.

The secondary xylem is completely lignified and is composed of scattered vessels and fibres with a large amount of xylem parenchyma. The vessels are up to about 60 μm in diameter and have moderately thickened walls; they are bordered pitted or reticulately-thickened. The xylem fibres are shorter than the fibres of the pericycle and they have fewer pits. The cells of the xylem parenchyma are longitudinally elongated and have thickened walls with numerous simple or bordered pits; the end walls are frequently oblique or slightly tapering (Figs 2·9, 3·5).

The pith is composed of thin-walled parenchymatous cells surrounding a central hollow which occupies about half to two thirds of the total diameter. A number of the cells contain cluster crystals of calcium oxalate.

STIPULE

Macroscopical characters

A membranous, sheathing stipule is present at each node; it is approximately triangular, measuring about 0·2 to 0·4 mm in height increasing to 0·5 to 0·7 mm at the highest point (Fig. 3·8a and b). Each stipule shows a fine, reticulate venation and at the base the inner surface is finely pubescent.

Histological characters

The outer epidermis is composed of thin-walled cells, elongated in surface view; the anticlinal walls are slightly sinuous and the cuticle is strongly striated (Fig. 3·6). Papillae, glandular trichomes and stomata are absent except at the extreme base, where a few papillae and stomata occur (Fig. 3·7). The inner epidermis is composed of cells with thinner walls than those of the outer epidermis and the cuticle is less markedly striated; each cell is about three or four times as long as broad when seen in surface view and the anticlinal walls are very finely wavy or minutely beaded (Fig. 3·10). Glandular trichomes are abundant on the inner epidermis and they show wide variation in size and form; some are similar to those occurring on the leaf, in others the stalk is unicellular and occasionally the multicellular head is much elongated and composed of many cells (Fig. 3·11). Abundant cluster crystals of calcium oxalate are present in the mesophyll, particularly in the cells near the veins; a few prisms also occur (Fig. 3·7).

INFLORESCENCE

Macroscopical characters

The inflorescence is cymose and the white or pinkish flowers occur in panicles. Each flower is small, about 6 mm is diameter when fully opened, and composed of five petaloid segments surrounding three styles and eight stamens. The ovary is superior and unilocular with a single ovule. The flowers are dimorphic, some having long stamens and short styles and others having short stamens and long styles. The fruits are three-sided, sharply angled and dark brown when ripe; they protrude from the perianth and are about 5 mm long.

Histological characters

Petaloid segments. The outer epidermis is composed of small, thin-walled cells, polygonal in surface view, each extended to form a small papilla; in the basal region the papillae are rounded and they become gradually more pointed towards the apex.

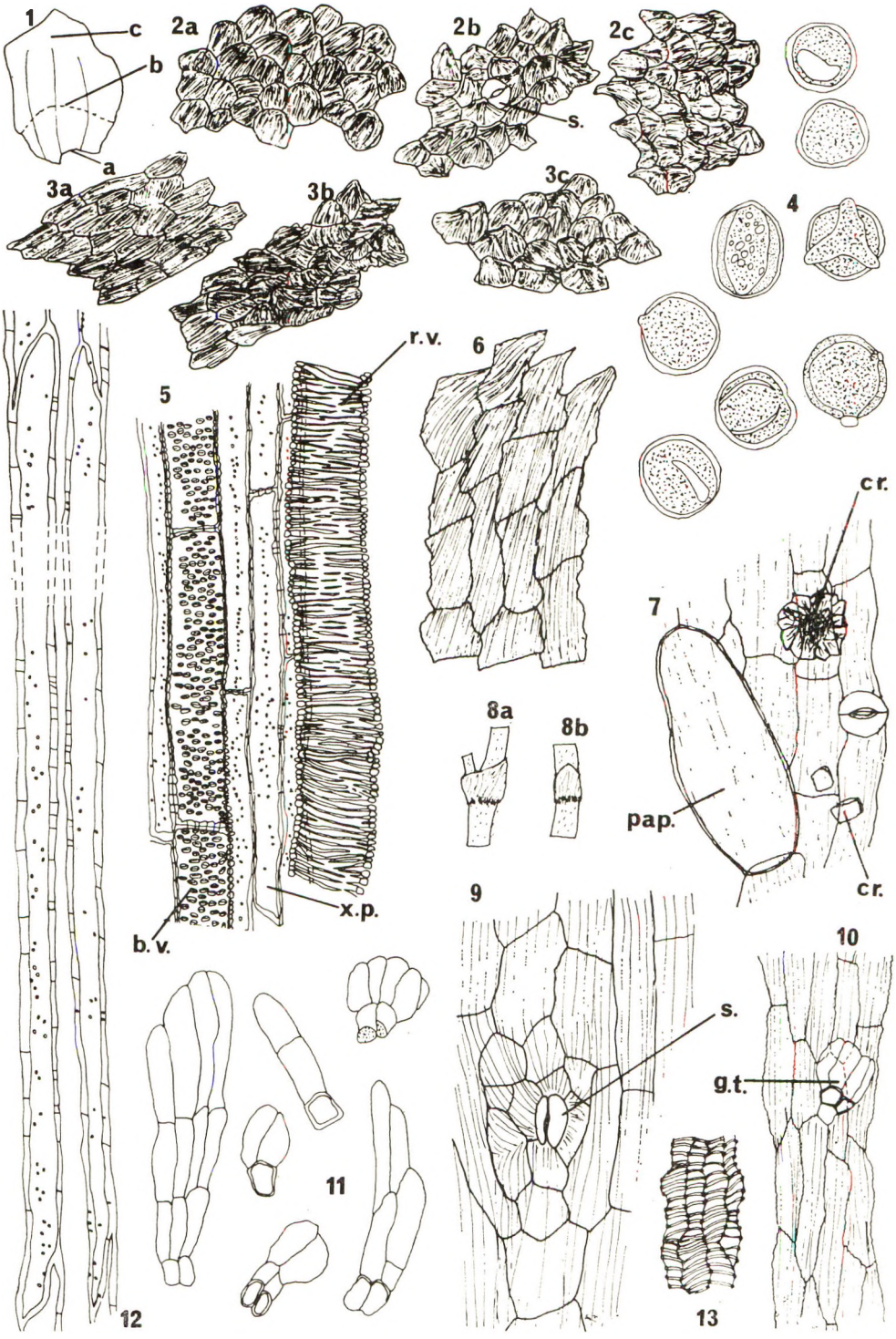

FIG. 3. *Fagopyrum esculentum*, stem and inflorescence. 1, diagram of a perianth segment × 6; 2a, 2b and 2c, outer epidermis and 3a, 3b and 3c, inner epidermis, of a perianth segment at the positions a, b and c marked on 1, in surface view; 4, pollen grains; 5, longitudinal section of the xylem of the stem; 6, outer epidermis near the apex of the stipule in surface view; 7, outer epidermis near the base of the stipule in surface view; 8a and 8b, diagrams of the stem showing the ochreate stipule, × ½; 9, epidermis of the stem in surface view; 10, inner epidermis of the stipule in surface view; 11, glandular trichomes from the inner epidermis of the stipule; 12, pericyclic fibres from the stem; 13, fibrous layer of the anther in surface view. All (except 1 and 8) × 220. b.v. = bordered pitted vessel; cr. = calcium oxalate crystals; g.t. = glandular trichome; pap. = papilla; r.v. = reticulately-thickened vessel; s. = stoma; x.p. xylem parenchyma.

The cuticle, which is continuous over the papillae, is strongly striated; very occasional stomata are present (Fig. 3·2). The cells of the inner epidermis show more variation than those of the outer epidermis; in the basal region they are axially elongated in surface view and are not papillose (Fig. 3·3a) whilst in the apical region the cells are polygonal and extended to form papillae (Fig. 3·3c); the transition from non-papillose to papillose cells occurs at approximately one third of the distance from the base (Fig. 3·1, 3·3b). As on the outer epidermis, the cuticle is strongly striated in all regions but stomata are absent.

Pollen grains. The pollen grains are spherical to ovoid with three pores, which are frequently indistinct, and a strongly pitted exine. Germinal furrows are not visible but occasional splits are seen in the exine in some of the grains (Fig. 3·4). Those from the flowers with short styles are about one-fifth larger than those from the long-styled flowers (Stevens, 1912); they measure 32·4–**42·5**–50·4 μm in diameter (long-styled flowers) and 43·2–**50·0**–57·6 μm in diameter (short-styled flowers).

SUMMARY

The microscopical characters of *Fagopyrum esculentum* Moench of value in identifying the aerial parts of the plant in the broken or powdered condition are as follows:

(a) The epidermis of the leaves; in surface view the cells of the upper epidermis with slightly sinuous walls and those of the lower epidermis with markedly sinuous walls; the cuticle over the upper epidermis strongly striated; anisocytic and occasional anomocytic stomata present on both epidermises but more numerous on the lower epidermis; glandular trichomes, composed of a biseriate stalk with two or four cells and a multicellular head, fairly abundant on both surfaces; covering trichomes absent but elongated papillae present, particularly on the upper surface over the veins. In transverse section the leaves show a dorsiventral structure with a two-layered palisade.

(b) The cluster crystals of calcium oxalate up to 45 μm in diameter and fewer prisms, up to 15 μm long, in the parenchymatous tissues.

(c) The lignified fibres, measuring up to 1·5 cm in length, in the pericycle and xylem of the stem.

(d) Lignified, reticulately-thickened and bordered pitted vessels up to 60 μm in diameter, associated with lignified xylem parenchyma, in the stem and petiole.

(e) The membranous stipules, composed of very thin-walled cells covered with a striated cuticle; glandular trichomes similar to those occurring on the leaves (but frequently larger) abundant on the inner epidermis, but absent from the outer epidermis.

(f) The perianth segments composed of small, straight-walled cells with a striated cuticle, most of the cells papillose.

(g) The sub-spherical pollen grains with three pores and a finely warty exine, 32·4–**42·5**–**50·0**–57·6 μm in diameter.

Acknowledgements

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LETTERS TO THE EDITOR

Resistance to tetrodotoxin in the isolated ileum of the rat

Su & Bevan (1970) reported the paradoxical finding that nicotine-induced contraction of the isolated pulmonary artery, was resistant to concentrations of tetrodotoxin (TTX) which inhibited neuronally-induced contractile responses to transmural electrical stimulation. They attributed the responses to nicotine or to transmural stimulation to release of noradrenaline. I now report another isolated smooth muscle preparation, the rat ileum, in which nicotine-induced contractions were not wholly blocked with TTX.

Strips of ileum from Male Charles River rats, 350–450 g, were suspended in muscle baths containing Tyrode solution at 36° and aerated with carbon dioxide in oxygen, under 1 g tension (Goldenberg, 1969). Responses to nicotine salicylate and to acetylcholine (chloride) were recorded isometrically. TTX (Calbiochem) or other antagonists were left in contact with the tissue for 10 min before adding agonist. All concentrations of drugs refer to final concentrations of their salts.

Transmural electrical stimulation was with one platinum electrode within the ileum lumen and the other surrounding the tissue. Stimuli were at 80V, 0·2 ms duration, at 3, 10 and 30 Hz. A pair of such stimuli 2 min apart, each of 5 s, was delivered at each frequency, and the mean of the two contractions recorded. An antagonist drug was left in contact with the ileum for 10 min before transmural stimulation was begun.

Nicotine, 1×10^{-5} g/ml, and acetylcholine, 5×10^{-8} g/ml, gave nearly equivalent biphasic contactile responses: a primary spike-like contraction followed by a slower progressive one (Fig. 1a). Procaine, 3×10^{-6} g/ml, elicited an 81 and a 64% inhibition of the primary and secondary contractile response to nicotine, in 10 experiments. The primary and secondary contractions in response to acetylcholine were slightly but significantly (paired *t*-test) inhibited, 14 and 7% respectively, by procaine. TTX, 1×10^{-7} g/ml, elicited a slight (21%) but significant reduction of the primary nicotine- induced contraction in 8 experiments (Fig. 1b). At 1×10^{-6} g/ml, TTX failed

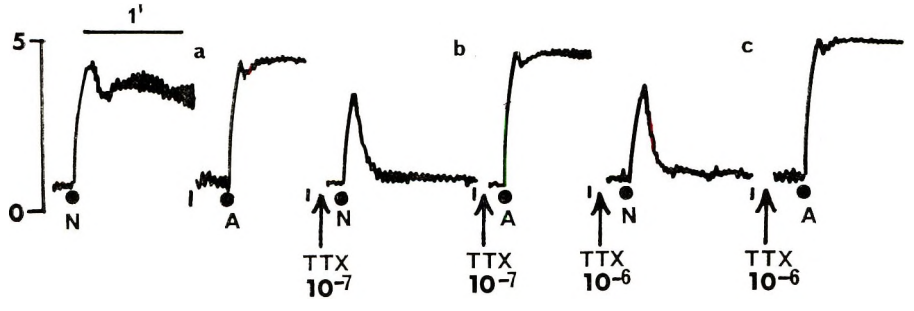

FIG. 1. Effect of nicotine (N) and acetylcholine (A) on the isolated rat ileum. (a) Control biphasic contractile responses to 1×10^{-5} g/ml of N and to 5×10^{-8} of A. (b) Responses to N and to A after 1×10^{-7} g/ml of tetrodotoxin (TTX). (c) Responses to N and to A after 1×10^{-6} g/ml of TTX. Note the resistance of the nicotine-induced primary spike-like contraction to complete blockade while the secondary contractile response was abolished. Contractile responses to A were not affected by TTX. Dot indicates when agonist was added to the bath; short vertical line represents washout of agonist or agonist-antagonist. Time mark, 1 min.

to cause any greater inhibition of the primary nicotine contraction than that observed at the lower concentration (Fig. 1c). TTX, 1×10^{-7} and 1×10^{-6} g/ml, did not inhibit the biphasic contractile response to acetylcholine in 6 strips (Fig. 1b and c).

Transmurally-induced contractions of the rat ileum were much, but not completely, diminished by hexamethonium, 1×10^{-4} g/ml. On the other hand, TTX, 1×10^{-7} g/ml, abolished contractions at all frequencies of such stimulation, in 6 experiments.

Nicotine is a ganglionic stimulant, but there have been many experiments which show the drug to act at presynaptic nerve endings where neurotransmitter substances are released (Trendelenburg, 1965: Chiou & Long, 1969; Bhagat, 1970). In the rat ileum, nicotine, at a maximal concentration, may stimulate the intrinsic nerve supply in at least two different sites. At ganglia, nicotine excites postsynaptic chemoreceptor sites and elicits excitatory postsynaptic potentials; nicotinic receptors on postganglionic nerve endings, on the other hand, are directly activated by nicotine to release acetylcholine, resulting in the primary spike-like contraction. Procaine nearly abolished the contraction and may act at both sites. TTX caused slight but not dose-related antagonism of the spike-like contraction, this inhibitory effect apparently taking place at neural sites, such that postsynaptic potentials were blocked (an effect, that is, similar to the complete blockade of transmurally-induced contractions of the rat ileum by TTX). That portion of the spike-like contraction which was resistant to TTX blockade may have been evoked by an "explosive" liberation of acetylcholine resulting from activation by nicotine of receptors in the neural endings. Nicotine stimulation, that is, unlike transmural stimulation, involves presynaptic receptor activation in the nerve endings, with sudden release of acetylcholine for a postsynaptic contractile effect. Katz & Miledi (1969) reported that the TTX-resistant portion of tetraethylammonium activity of the giant synapse in the squid stellate ganglion was localized in the terminal parts of the presynaptic axons.

The "explosive" effect, as described by Löffelholz (1970) for the release of noradrenaline from adrenergic terminal fibres by nicotinic drugs in the rabbit isolated heart, may be responsible for the nicotine-induced TTX-resistant contraction of the pulmonary artery reported by Su & Bevan (1970).

The secondary contractile response to nicotine was sensitive to TTX blockade. In this situation, nicotine activity may be ascribed to ganglionic excitation (either directly or indirectly through acetylcholine released preganglionically), with subsequent propagation of nerve impulses in the intrinsic nerve plexuses. Thus TTX, which interferes with conduction of nerve impulses, inhibits the secondary contractile response to nicotine just as TTX abolished the responses to transmural stimulation.

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The effect of β-phenethylamine on noradrenaline concentrations in guinea-pig brain

β-Phenethylamine (PE) is an indirectly acting sympathomimetic amine which crosses the blood brain barrier to cause central nervous system (c.n.s.) stimulation (Mantegazza & Riva, 1963). PE occurs *in vivo* in human urine (Asatoor & Dalgliesh, 1959), rabbit brain and other tissues; (Najakima, Kakimoto & Sano, 1964; Jackson & Temple, 1970). On animals it exerts a c.n.s. stimulant effect resembling that of amphetamine (Mantegazza & Riva, 1963; Saavedra & Fischer, 1970), and like amphetamine causes marked depletion of noradrenaline in heart and brain (Jonsson, Grobecker & Holtz, 1966) and some depletion of dopamine (Fuxe, Grobecker & Jonsson, 1967). PE may serve as a neurohumor *in vivo* (Nakajima & others, 1964; Mantegazza & Riva, 1963), and evidence has been presented (Saavedra & Fischer, 1970) that PE and tryptamine may play opposing roles in the c.n.s. in much the same way as has been proposed for noradrenaline and 5-hydroxytryptamine.

With these considerations in mind, I now report the effect of PE on noradrenaline and dopamine concentrations in guinea-pig brain.

Guinea-pigs of either sex (400–550 g) were killed by decapitation and the brain immediately removed and extracted with 0·4M perchloric acid. Noradrenaline and dopamine were extracted (Anton & Sayre, 1962) and the former estimated according to Haggendal (1963) and the latter according to Anton & Sayre (1964). Recovery of both amines was between 70 and 80%. PE was administered intraperitoneally as the hydrochloride, dissolved in normal saline to give a dose volume of 1 ml/100 g. Saline was used for control injections. After injection and until death, animals were kept isolated in quiet surroundings. Each brain was assayed individually and the values are uncorrected for % yield.

Animals received one dose of either 100 or 200 mg/kg of PE and were killed at various intervals after injection. An effect was observed on brain noradrenaline concentration within 15 min. Peak depletion was one h after injection and recovery to control values was within 24 h (with the high dose) (Fig. 1). The depletion of

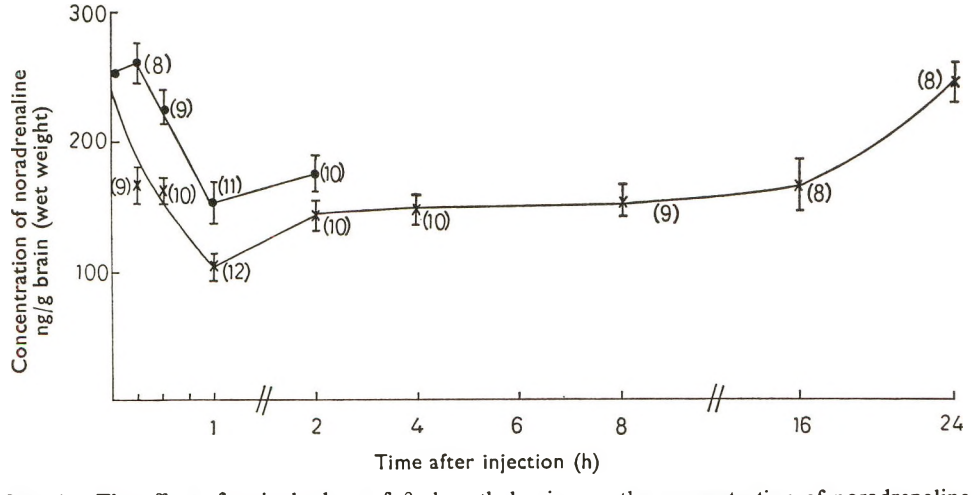

FIG. 1. The effect of a single dose of β-phenethylamine on the concentration of noradrenaline in guinea-pig brain. Animals were given a single dose of either 100 or 200 mg/kg, i.p. and a number killed at various time intervals after this injection. The vertical bars represent the standard errors of the means. The values in brackets are the number of animals in each group. ●, 100 mg/kg. ×, 200 mg/kg.

noradrenaline was dose-dependent, and at the highest dose, marked behavioural changes, including licking, and clonic convulsions in some animals, were observed. These changes were maximal 1 h after injection of PE. There was no detectable change in brain dopamine levels.

Repeated doses of PE (100 mg/kg) at 1 h intervals to another group of guinea-pigs showed that maximum noradrenaline depletion (representing 74% of total noradrenaline stores) occurred after the third injection (total dose of 300 mg/kg PE at 100 mg/kg h^{-1}), and that the concentration after three injections was not significantly different from that after six injections ($P > 0{\cdot}1$).

The findings are in agreement with those of Jonsson, Grobecker & Holtz (1966), that PE in rats causes a marked depletion of brain noradrenaline concentrations, without significantly affecting those of dopamine, although Fuxe, Grobecker & Jonsson (1967) found small but significant changes in brain dopamine concentrations in rats. The doses of PE I used were high but it is rapidly broken down by monoamine oxidase (Blaschko, 1952; Mantegazza & Riva, 1963). The depletion of brain noradrenaline was accompanied by behavioural changes which appeared maximal when maximum noradrenaline depletion had occurred (at 1 h). The depletion produced resembled that caused by amphetamine (Moore & Lariviere, 1963), except that maximal depletion took 1 h compared to 4 h with amphetamine, and the effect lasted 24 h compared to 7 days with amphetamine. Maximum noradrenaline depletion of 74% agreed well with the depletion of approximately 70% in rats found by Jonsson & others (1966).

Measures of spontaneous motor activity in mice suggests that peak activity after intraperitoneal injection of PE is reached in 5–7 min (Nakajima & others 1964; Jackson & Temple, unpublished observations), and a return to normal levels of motor activity occurs within 15 min. In spite of the species difference, these reports suggest that there may not be a clear correlation between spontaneous coordinated motor activity and noradrenaline depletion by PE, and that other factors, one of which may be a direct component of action, may be operating, in the same way as has been suggested for the cns effects of amphetamine (Smith, 1963, 1964). The work of Saavedra & Fischer (1970) and my preliminary experiments seem to support such a hypothesis.

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Maintenance of noradrenaline in neuronal cell bodies and terminals: effect of frequency of stimulation

Upon the arrival of action potentials in noradrenergic nerve terminals noradrenaline is released (Brown, 1960; Haefely, Hürlimann & Thoenen, 1965), much of the released noradrenaline is retrieved (Blakeley, Brown & Geffen, 1964), and noradrenaline synthesis is accelerated (Alousi & Weiner, 1966). The results of these studies suggest that steady state levels of noradrenaline in the nerve terminals are maintained by local synthesis and by reuptake. The relative importance of these two processes in resting and active neurons remains controversial (Malmfors, 1964; Hedqvist and Stjarne, 1969). For example, in nerve terminals, synthesis of noradrenaline is believed to be of prime importance at low frequencies of stimulation while reuptake of this amine predominates at high frequencies (Bhagat and Friedman, 1969). On the other hand, in noradrenergic nerve cell bodies noradrenaline stores appear to be maintained by synthesis independent of neuronal activity (Moore & Bhatnagar, 1970). We have examined further the roles of synthesis and uptake in the maintenance of noradrenaline stores in both cell bodies and terminals of noradrenergic nerves during different frequencies of stimulation.

Experiments were conducted in 1·6–3·5 kg cats anaesthetized with Dial-Urethane. Superior cervical ganglia represented the cell bodies, and salivary glands and nictitating membranes the terminals of peripheral noradrenergic neurons. Preganglionic fibres were decentralized and stimulated unilaterally at low and high frequencies of stimulation (2 or 10 Hz for 30 s/min for 3 h). The upper limit of "physiological" frequency of impulses is thought to be 10 Hz (Folkow, Häggendal & Lisander, 1967). The viability of the ganglionic transmission was monitored by recording contractions of the nictitating membranes during preganglionic stimulation. Synthesis of noradrenaline was inhibited by α-methyltyrosine (Spector, Sjoerdsma & Udenfriend, 1965) infused through both common carotid arteries at the rate of 0·1 mg/min for 3 h. This dosage schedule had been found by us to block the synthesis of [^{14}C]noradrenaline from [^{14}C]tyrosine in ganglia, salivary glands and nictitating membranes. Control animals received infusions of 0·9% NaCl. Saline and α-methyltyrosine infusions were begun at the start of the preganglionic stimulation. Re-uptake of noradrenaline was blocked by intravenous injections of desipramine, 2 mg/kg of which were administered at the start of stimulation and another 1 mg/kg 2 h later; a dose found by us to block the uptake of [^{3}H]noradrenaline in submaxillary salivary glands and nictitating membranes but not in ganglia. At the end of the experiment, tissues were removed and analysed for noradrenaline. Submaxillary salivary glands (frozen in liquid nitrogen and pulverized) and ganglia were homogenized in 0·4N $HClO_4$. Nictitating membranes with orbital attachments were cut into small pieces and allowed to stand in 0·4N $HClO_4$ overnight. The noradrenaline in perchloric acid extracts was isolated by alumina adsorption and analysed by the trihydroxyindole method as described by Moore & Rech (1967).

The effects of α-methyltyrosine, desipramine, and different frequencies of stimulation on the noradrenaline contents of superior cervical ganglia, salivary glands and nictitating membranes are summarized in Table 1.

We have previously reported that in unstimulated preparations α-methyltyrosine reduces the noradrenaline concentrations in cell bodies but not in terminals, while desipramine has no effect on the noradrenaline content in either cell bodies or terminals (Bhatnagar & Moore, 1970). Although the present experiments were not specifically designed to compare drug effects in non-stimulated preparations, the same pattern was apparent. That is, when the effects of drugs in all non-stimulated tissues

Table 1. *Effects of α-methyltyrosine, desipramine and electrical stimulation at 2 and* 10 Hz *on tissue noradrenaline contents.*

Treatment	Frequency of stimulation (Hz)	Superior cervical ganglia (μg/g)			Submaxillary salivary glands (μg/g)			Nictitating membranes (μg/membrane)		
		N	S	S/N × 100	N	S	S/N × 100	N	S	S/N × 100
Saline	2	5·96 ± 0·97	6·92 ± 0·93	116	1·27 ± 0·16	1·02 ± 0·09	81	1·37 ± 0·22	1·28 ± 0·25	93
	10	8·25 ± 0·81	7·50 ± 0·34	91	1·70 ± 0·30	0·72 ± 0·03	42†	1·22 ± 0·08	0·90 ± 0·09	74†
α-Methyltyrosine	2	3·32 ± 0·73	3·50 ± 0·34	105	1·89 ± 0·23	1·07 ± 0·17	57*†	1·01 ± 0·17	0·76 ± 0·17	75
	10	3·75 ± 0·39	3·37 ± 0·52	90	1·29 ± 0·10	0·16 ± 0·25	12*†	1·08 ± 0·13	0·52 ± 0·03	48*†
Desmethylimipramine	2	6·59 ± 1·14	6·40 ± 1·36	97	1·47 ± 0·31	1·04 ± 0·22	71†	1·24 ± 0·12	1·04 ± 0·18	84
	10	9·26 ± 1·08	8·38 ± 1·00	90	1·63 ± 0·16	0·39 ± 0·05	24*†	1·63 ± 0·27	0·98 ± 0·19	60*†
α-Methyltyrosine and desmethylimipramine	2	2·57 ± 0·54	2·60 ± 0·77	101	1·30 ± 0·17	0·58 ± 0·08	45*†	0·92 ± 0·12	0·80 ± 0·18	87
	10	2·06 ± 0·12	2·82 ± 0·66	137	1·70 ± 0·18	0·04 ± 0·00	2*†	1·26 ± 0·11	0·47 ± 0·10	37*†

Values in non-stimulated (N) and in stimulated (S) tissues represent the mean content of noradrenaline ±1 s.e. determined in 3–7 separate experiments.
*—Significantly different from corresponding saline controls ($P < 0.05$).
†—Significantly different from 100% ($P < 0.05$).

were collated it was clear that α-methyltyrosine, but not desipramine, significantly reduced the noradrenaline content of ganglia (saline 6·88 ± 0·68 μg/g; α-methyltyrosine, 3·49 ± 0·45 μg/g; desipramine, 8·08 ± 0·87 μg/g). On the other hand, neither desipramine nor α-methyltyrosine significantly altered noradrenaline contents in salivary glands and nictitating membranes.

Since there was much less variation in the tissue concentration of noradrenaline between the right and left sides of the same cat than there was between cats, experiments on the effects of stimulation were designed so that the contralateral tissues of the same cat served as controls. Preganglionic stimulation alone or in the presence of α-methyltyrosine or desipramine, or both, did not alter the noradrenaline content of ganglia. That is, the noradrenaline concentrations in stimulated ganglia expressed as a percentage of noradrenaline in non-stimulated ganglia were not statistically different from 100%. Thus, noradrenaline in ganglia is maintained by synthesis independent of the frequency of neuronal activity. In contrast to the ganglia, preganglionic stimulation alone reduced noradrenaline concentrations in salivary glands; stimulation at 2 and 10 Hz reduced noradrenaline content to 81 and 42% of the corresponding unstimulated gland. α-Methyltyrosine caused a further reduction in the stimulus-induced decline of noradrenaline at both 2 and 10 Hz (to 57 and 12% respectively), whereas desipramine increased the depletion of noradrenaline only at the higher frequency of stimulation. The combination of desipramine and α-methyltyrosine enhanced the noradrenaline depletion at 2 Hz and caused an almost total depletion at 10 Hz. The effects of preganglionic stimulation, desipramine and α-methyltyrosine in nictitating membranes were qualitatively similar to those seen in salivary glands with the effects being significant only at the higher frequency of stimulation and the changes in noradrenaline concentrations being less pronounced. Thus, in nerve terminals, synthesis partially maintains noradrenaline concentrations during high and low frequencies of stimulation. Reuptake, on the other hand, appears to play a significant role only at higher frequencies of stimulation.

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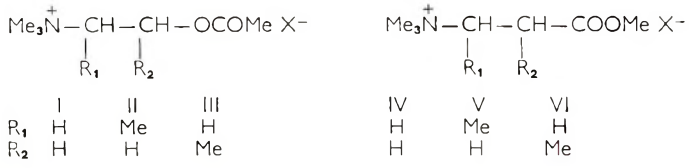
The muscarinic and nicotinic activities of some esters related to acetylcholine

Schueler & Keasling (1951) reported that the reversed carboxyl analogues of acetyl-α-methylcholine and acetyl-β-methylcholine, namely methyl 3-dimethylaminobutyrate methiodide (V) and methyl 3-dimethylamino-2-methylpropionate methiodide (VI), possessed less than one ten-thousandth of the muscarinic activity of their respective acetylcholine analogues (II and III). Methyl 3-dimethylaminopropionate methiodide (IV), which is the reversed carboxyl analogue of acetylcholine (I), is reported to possess at least one-tenth the activity of, and at most to be equipotent with, acetylcholine (Bass, Schueler & others, 1950; Barrass, Brimblecombe & others, 1968). In view of this high activity shown by the ester (IV) compared with compounds V and VI, we have synthesized and re-examined the muscarinic and nicotinic activities of all three esters (IV, V and VI).



	I	II	III	IV	V	VI
R_1	H	Me	H	H	Me	H
R_2	H	H	Me	H	H	Me

For the preparation of esters IV-VI, equimolar quantities of anhydrous dimethylamine were reacted with solutions of methyl acrylate, methyl crotonate or methyl methacrylate respectively, in anhydrous methanol. The cooled amine solution was slowly added with stirring to the cooled solution of the ester and the resulting mixture maintained at 0° for 2 h, then maintained at room temperature for 24 h. The resulting tertiary amines were purified by fractional distillation and characterized by analysis, and by their nmr and infrared spectra.

The methiodides (IV-VI) were obtained by dissolving each tertiary amine in acetone and adding an excess of a solution of methyl iodide in acetone. The purified products were characterized by their infrared, nmr and mass spectra.

The muscarinic activity of each compound was examined on the blood pressure of the anaesthetized rat and on the guinea-pig ileum. The molar potency of each compound, relative to acetylcholine, was determined in the absence and in the presence of a ganglion blocking agent to exclude possible interference from any nicotinic activity the compounds might have. The results are summarized in Table 1. All the compounds were less active than acetylcholine but compounds V and VI were much more active than reported previously (Schueler & Keasling, 1951). Compounds II (acetyl-α-methylcholine), IV and V showed a marked increase in potency in the presence of the ganglion blocking agents. In the rat, hyoscine methiodide (5 mg/kg) intravenously abolished the fall in blood pressure caused by all compounds. Similarly, the contractile effect of the compounds on the guinea-pig ileum was abolished by hyoscine methiodide (0·1 μg/ml). These results indicate that the compounds have a muscarinic action.

The nicotinic activity of the compounds was studied on the blood pressure of the rat pre-treated with hyoscine methiodide (5 mg/kg) intravenously and on the frog rectus muscle. The results are in Table 2. Compounds II, IV and V possessed significant nicotinic activity on both test preparations; compounds III and VI had little or no nicotinic activity as previously reported (Schueler & Keasling, 1951; Beckett, Harper & Clitherow, 1963). A comparison of the muscarinic and nicotinic activities of the

Table 1. *The muscarinic activities of some acetylcholine analogues and related esters.* Molar potencies (±s.e.) were determined relative to acetylcholine = 1·00.

Compound	Rat blood pressure n = 4	Rat blood pressure (Pre-treated with pentolinium tartrate (5 mg/kg)) n = 2	Guinea-pig ileum n = 4	Guinea-pig ileum (In presence of hexamethonium, 100 μg/ml) n = 2
II	0·02 (±0·01)	0·04	0·03 (±0·01)	0·05
III	0·40 (±0·11)	0·40	0·86 (±0·17)	0·88
IV	0·11 (±0·03)	0·50	0·30 (±0·15)	0·30
V	0·11 (±0·01)	0·03	0·05 (±0·03)	0·06
VI	0·02 (±0·01)	0·01	0·03 (±0·01)	0·02

Table 2. *The nicotinic activities of some acetylcholine analogues and related esters.* Molar potencies (±s.e.) were determined relative to acetylcholine = 1·00.

Compound	Frog rectus muscle n = 4	Rat blood pressure (Pre-treated with hyoscine methiodide (5 mg/kg)) n = 4
II	0·17 (±0·06)	0·80 (±0·10)
III	<0·001	<0·01
IV	0·97 (±0·31)	0·85 (±0·07)
V	0·12 (±0·03)	0·75 (±0·17)
VI	<0·001	<0·01

compounds suggested that II, IV and V have more specific nicotinic action than acetylcholine. However, the specificity was much less marked with compound IV. It was noteworthy that methyl 3-dimethylaminobutyrate methiodide (V) was slightly more potent than acetyl-α-methylcholine (II).

The activities shown by compounds V and VI in our experiments were much higher than those reported by Schueler & Keasling (1951). Perhaps these workers were using impure compounds; we were unable to obtain pure compounds by their method of preparation.

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In vitro study of mutual antagonism between α- and β-adrenoceptor blocking agents

Many unrelated drugs cause a reappearance of the adrenaline-pressor response that is blocked by α-adrenoceptor blocking agents (Osswald, 1969). An antagonism of α-receptor blockers by β-receptor blocking agents, *in vivo* and *in vitro*, has been widely reported (see, Krell & Patil, 1969) though the mechanism of this interaction is not clear.

One explanation assumes that blockade of β-adrenoceptors after administration of α-receptor blockers mainly unmasks residual α-effects (Garrett, Malafaya-Baptista & Osswald, 1966; Nickerson, 1970). The concept finds a strong support in the findings that the antagonism of α- by the β-adrenergic blocker is seen only if a test preparation exhibiting functionally opposed α- and β-adrenoceptors is used together with an agonist that excites both the receptor species. Also, the pressor activity of a sympathomimetic drug exerted at a time when a β-blocker has antagonized α-receptor blockade never quite reaches its control magnitude.

On testing this concept *in vitro*, however, we found that pronethalol and tolazoline antagonize each other even though relatively pure agonists, isoprenaline and phenylephrine, were used with rabbit isolated ileum and perfused heart of frog (ambient temperature, 18°–22°). In these preparations the sympathomimetic excitation of either α- or β-receptor results in a similar overt effect (Furchgott, 1960; Buckley & Jordon, 1969).

Phenylephrine produced a concentration-related relaxation of the rabbit ileum the curves for which were unaffected by pronethalol (5 ng/ml, n = 5, Fig. 1) but were parallelly shifted to the right by tolazoline (0·5 μg/ml); pronethalol now shifted the curves back to their control position on the concentration-axis despite the presence of tolazoline in the bath (n = 11, Fig. 1). Again, tolazoline (0·5 μg/ml, n = 7) which had no major effect on the relaxant action of isoprenaline (Fig. 1) totally reversed the blockade of isoprenaline provoked by pronethalol (5 ng/ml). A similar mutual antagonism between α- and β-adrenergic blockers was reported *in vivo* by Ahlquist & Levy (1959) who studied responses of canine ileum.

The inotropic effect of agonists on frog heart (perfused as described by Buckley & Jordon, 1969) was recorded using a light spring lever. Pronethalol (100 ng/ml of perfusion fluid) nearly blocked the inotropic effect of submaximal doses of isoprenaline (2 to 5 μg, tested at 7 min intervals). Within 5 to 20 min of perfusion with fluid to which tolazoline (20 μg/ml) was also added the response to isoprenaline recovered up to 80 to 100% of the control (n = 10); tolazoline alone had no significant effect on the response to isoprenaline. The blockade, however, supervened again totally (n = 3) or partially (n = 7) in the following 30 min of continued perfusion. Doubling the strength of tolazoline in the fluid at this stage again produced a comparable recovery of response to isoprenaline. In another series of experiments (n = 6), pronethalol (75 ng/ml of fluid) did not alter the inotropic effect of submaximal doses of phenylephrine (100 to 275 μg, tested at 10 min intervals), but partially reversed the block provoked by tolazoline (10 μg/ml in the fluid). At the time of maximal recovery, the response to phenylephrine was 50 to 70% of the control.

Allowing that isoprenaline stimulated the α-receptors at a time when pronethalol had blocked the β-receptors, tolazoline was expected to block the residual relaxation of the ileum or the stimulation of the heart caused by isoprenaline. Hence, recovery from pronethalol-induced blockade due to tolazoline, occurring in a test sytem exhibiting parallelly functioning α- and β-receptors should have some basis other than that proposed by Garrett & others (1966). The contention is further supported by the finding that action of phenylephrine, blocked by tolazoline, reappeared totally

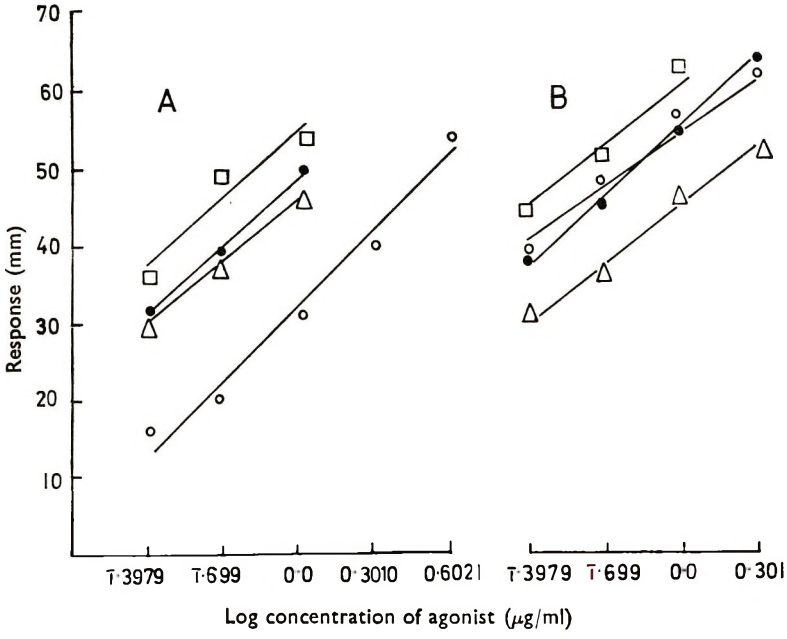

FIG. 1. Rabbit isolated ileum (Tyrode solution at 33° ± 1°, gassed with 5% CO_2 in O_2). Concentration-response curves for (A) isoprenaline HCl (tested at 6 min intervals) and (B) phenylephrine HCl (tested at 10 min intervals) in absence and in presence of antagonists added 5 min before. —●— Control. —△— Tolazoline 0·5 μg/ml. —○— Pronethalol 5 ng/ml. —□— Pronethalol 5 ng and tolazoline 20 μg/ml.

(ileum experiments) or partially (heart experiments) after administration of pronethalol.

Pronethalol and tolazoline (in concentrations used in this study) did not sensitize the tissues to phenylephrine and isoprenaline, respectively. This seems to exclude further that sensitization by one blocker of receptors spared from blocking activity of their regular, specific blocker (Osswald, 1960) can explain the observed antagonism between pronethalol and tolazoline.

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Antagonism of acetylcholine-induced bronchospasm by WG 253, a new sympathomimetic amine

WG 253, [erythro-(3,4-dihydroxyphenyl)(2-piperidyl) methanol hydrobromide], a member of a new series of sympathomimetic amines cyclized about the α-carbon atom gave longer protection against histamine-induced bronchospasm than did isoprenaline when administered by aerosol to human volunteers (Griffin & Turner, 1971). In addition, isoprenaline had much greater cardiovascular effects as measured by pulse rate, blood pressure and electrocardiographic changes.

A within-subjects double-blind comparison of aerosol preparations of isoprenaline, orciprenaline, salbutamol and WG 253 in protecting human volunteers against acetylcholine induced bronchospasm has now been made. Eight normal subjects of either sex (aged 20–36 years) took part, and the order of treatments within subjects was determined from two latin squares, the tests being carried out at intervals of 7 days. On each test day, two control measurements of FEV_1 were made on each subject using a Vitalograph. The subject then inhaled 500 μg of acetylcholine from a pressurized aerosol delivering 250 μg/puff. Measurements of FEV_1 were then made at 30 s after inhalation of acetylcholine and at 1 min intervals for 10 min. Two h after the first inhalation of acetylcholine, two further control measurements of FEV_1 were made, then the subject was given from an aerosol preparation one metered dose of isoprenaline (100 μg/dose), orciprenaline (750 μg/dose), salbutamol (100 μg/dose) or WG 253 (500 μg/dose), according to the randomization schedule. (The aerosol dispensers were not distinguishable one from another.)

Five min after receiving the bronchodilator aerosol the subjects were challenged again with 500 μg of acetylcholine, the FEV_1 being measured as previously. The changes in FEV_1 from baseline control values were determined for acetylcholine alone and for acetylcholine given after pretreatment with a bronchodilator aerosol. These values were subjected to a multivariate analysis of dispersion followed by calculation of T^2 for all contrasts between pairs of drugs (Smart, Sneddon & Turner, 1967; Cherrington & Smart, 1971).

The $\frac{1}{2}$ min values were significantly greater than zero, indicating that all drugs had reduced the fall in FEV_1 produced by acetylcholine. All the other values up to 10 min were significantly greater than zero, except for salbutamol which was not significantly different from zero after 6 min. There was no difference between the other treatments at any of the times measured.

Thus all four compounds exerted a significant protection against bronchospasm induced by acetylcholine within half a minute of inhalation, and the effect was maintained for at least 10 min for all drugs except salbutamol under the experimental conditions and in the doses used.

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A comparison of the effects of isoprenaline, WG 253 and salbutamol on the tension and rate of rabbit isolated atria

I have compared the effects of isoprenaline, WG 253 [erythro-(3,4-dihydroxyphenyl)-(2-piperdyl) methanol hydrobromide] and salbutamol on the rate and tension of isolated rabbit atria, and related these findings to the ratio for isoprenaline and salbutamol reported by Callum, Farmer & others (1969).

Pairs of atria were taken from six 500 g Dutch rabbits that had been killed by a blow on the back of the head and bled from the carotid arteries. The atria were in 70 ml of Ringer Locke solution gassed with 3% carbon dioxide in oxygen at 28°; recordings were made with isometric force-displacement transducers, Devices DC6 amplifiers and M2 recorders at a paper speed of 1 mm/s. After a control record of 30 s drug was added and the recorder was run for 120 s; the bath was then washed out three times at 3 min intervals. Intervals between doses were 15 min. Dose response curves were made for each drug using each pair of atria, a procedure followed for each of six experiments, in which drugs were added in a different order. The mean results of six experiments, in which the ratio of treated tension to control tension is plotted against the log dose, are shown in Fig. 1. The semi-log dose response

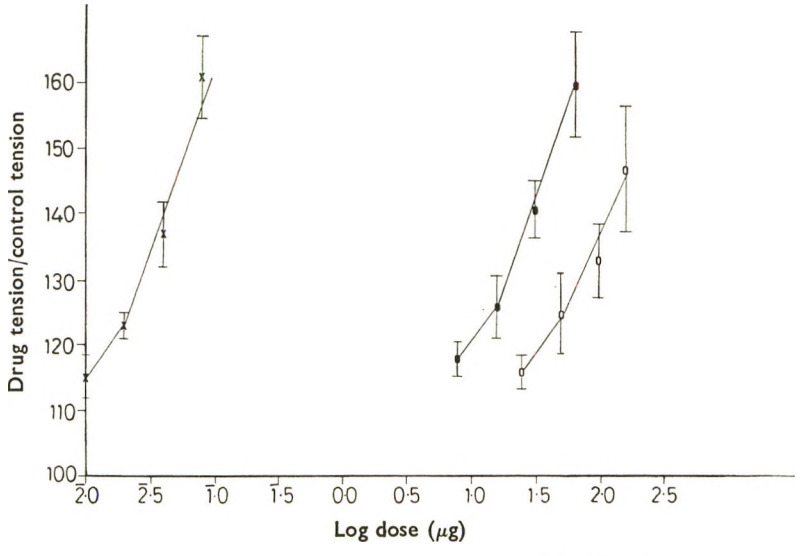

FIG. 1. Log dose-response curves to isoprenaline (×—×), WG, 253 (●—●) and salbutamol (○—○), obtained using rabbit isolated atria suspended in 70 ml, Ringer-Locke solution gassed with 97% oxygen and 3% carbon dioxide at 28°. Each point represents the mean ± standard error of six experiments.

curves are nearly parallel. From the curves, it was calculated that isoprenaline was about 720 times as potent as WG 253 and 2710 times as strong as salbutamol in its effect upon tension developed by the atria. Similar results were found when the ratio of treated rate to control rate was plotted against the log of the dose, the relative potencies being 1 to 790 to 2100. The relative potencies of isoprenaline and salbutamol compare well with those of Callum & others (1969) on guinea-pig atria tension, which were 1 to 2000.

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On the presence of histidine decarboxylase activity in rat forestomach

Recent reports on the presence of histidine decarboxylase (L-histidine-carboxy-lyase, EC 4.1.1.22) in the rat fundus (forestomach, thin membranous or ruminal portion) and the pylorus (glandular or muscular portion) have been contradictory. Radwan & West (1967) claimed preparations from the rat fundus to have many of the enzymatic properties of the specific histidine decarboxylase found in rat foetal liver and rat hepatoma; the enzymatic activity of the fundic preparations, however, was slightly inhibited by α-methyl histidine while preparations from the pylorus displayed many of the enzymatic properties of the aromatic-L-amino acid decarboxylase (EC 4.1.1.26). Håkanson & Owman (1966) and Aures, Håkanson & Schauer (1968) found that the ruminal portion had only a trace of the enzyme activity compared with the oxyntic gland area of the pyloric portion. Leinweber & Braun (1970) concluded that, in addition to the pylorus, the fundic portion also contains histidine decarboxylase activity which is resistant to most known inhibitors. The lack of response to inhibition was used to explain the failure to obtain a reduction of histamine formation *in vivo*. These authors also suggested that the activity of the fundic portion bore a striking similarity to that of the *Lactobacillus* enzyme purified by Rosenthaler, Guirard & others (1965). Kobayashi & Maudsley (1969) believed that the enzyme activity of the fundus was weak and in an insoluble form, and was present only in male rats. Isaac (1970) and Beavens, Horáková & Severs (1970) presented evidence from studies in germ-free rats that the fundus activity was of bacterial origin.

These contradictory communications prompted us to describe our early investigations on this subject which indicate that the non-enzymatic activity of the fundus offers an alternative explanation to these conflicting reports.

Adult, male Sprague-Dawley rats (CFE, Carworth Farms, New City, New York), 200 to 250 g, and fed Purina rat pellets *ad libitum* were killed by decapitation and the whole stomach immediately excised. The thin forestomach was separated from the muscular pyloric portion by cutting along the line of demarcation. Both portions were washed free of stomach contents in 0·9% cold saline and homogenized in an all-glass homogenizer with 3 volumes of cold saline. The homogenates were centrifuged at 35 000 *g* for 1 h at 4° and the resulting supernatant frozen in 2 ml aliquots at −20°, if not used immediately.

Histidine decarboxylase was assayed (Ellenbogen, Markley & Taylor, 1969). Unless otherwise noted, incubation mixtures contained 0·2 ml of 0·1M sodium phosphate buffer (pH 6·8), 0·2 ml of 1×10^{-5}M pyridoxal phosphate, 0·5 ml of enzyme, 0·1 ml of 1×10^{-2} M L-histidine-^{14}COOH (New England Nuclear or Calbiochem) and water to a final volume of 2·0 ml. After the addition of the enzyme and cofactor, the mixture was allowed to incubate for 10 min. The reaction was started by the addition of substrate, incubated at 37° for 1 h and corrected for a boiled enzyme control (boiled 30 min). All assays were made in duplicate and the results averaged.

Table 1. *Comparative properties of preparations from fundus and pylorus.*

Source of material	Treatment*	Activity (% of native material)
Fundus	Boiled	100
	Perchloric acid	92
	Dialysed	0
Pyloric	Boiled	17
	Perchloric acid	33
	Dialysed	70

* See text for details.

When incubation mixtures were corrected for boiled enzyme controls from the same rat stomach preparation, the following observations were made (Table 1): (1) Boiling the supernatants for 30 min usually did not decrease the activity of the fundus, and frequently produced an increase; the activity of the supernatant from the pyloric portion was decreased by boiling for 2 min and further boiling for up to 90 min caused no further drop in activity. (2) With preparations from the fundus, approximately 92% of the activity was recovered after precipitation in 5% perchloric acid and neutralization to pH 6·5, 10 min later; similar treatment markedly reduced the activity of the preparations from the pylorus. (3) Activity of the fundus was completely eliminated by dialysis whereas dialysis of the pylorus preparations caused a small decrease in activity.

With preparations from the pylorus, a linear response was obtained with enzyme amounts in excess of 10 mg and incubation times up to 2 h; a linear response could never be obtained beyond 1 mg of protein prepared from the fundus at a pH of 6·8 (Fig. 1). Activity was difficult to measure much below 1 mg of protein at pH 6·8 and only trace amounts were detectable at pH 5·6.

The above characteristics would indicate that much of the fundic activity could be non-enzymatic and is likely to be related to a heat-stable residual activity found adjacent to, or contaminated by, the pylorus. The studies by Aures & others (1968) on the

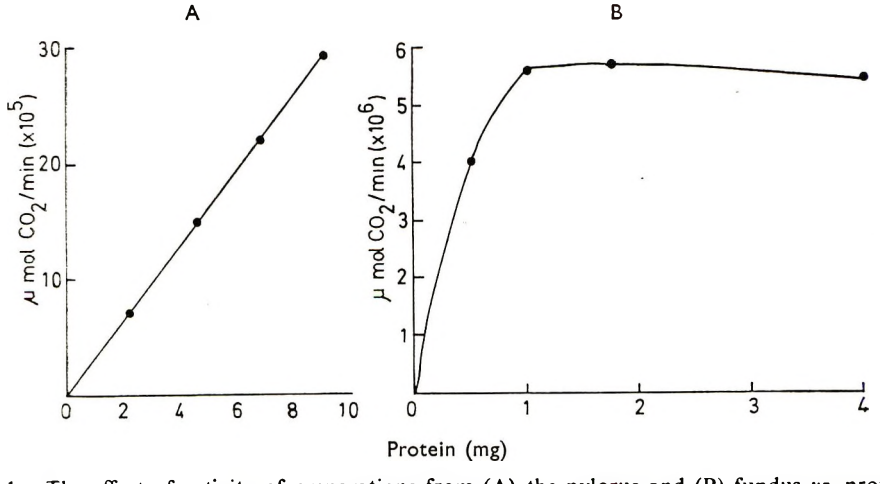

FIG. 1. The effect of activity of preparations from (A) the pylorus and (B) fundus *vs.* protein concentration. A 0·05–0·5 ml sample of 35 000 *g* supernatant was used as the source of "enzyme". Assay is described in text. Each point represents the average of 2 assays.

regional distribution of histidine decarboxylase indicate that enzyme activity occurs at the distal portion of the pyloric glandular area (oxyntic gland area) which is adjacent to the fundic portion. Thus, contamination by traces of the pyloric area could explain our occasional finding of some erratic activity in the forestomach. The highest specific activity observed with fundic preparations was 10% of that observed with material from the pylorus. The non-enzymatic decarboxylation could also explain the formation of nearly stoichiometric amounts of histamine and $^{14}CO_2$ observed by Leinweber & Braun (1970). The suggestion (Leinweber, 1968) that the forestomach activity is similar to that of the enzyme purified from *Lactobacillus* (Rosenthaler & others, 1965) is contradicted by the fact that cyanide does not inhibit the mammalian activity. The variable results seen by the different investigators were attributed to the variable amount of bacteria found in the stomach of the rats (Isaac, 1970; Beavens & others, 1970). The findings reported herein, however, offer an alternative explanation. Furthermore, decarboxylation catalysed by pyridoxal phosphate or pyridoxal itself occurs in the absence of any apoenzyme, and almost all of these reactions proceed by the same mechanism (Metzler, Ikawa & Snell, 1954; Snell, 1958). In addition, Lippmann (1969) has recently shown that production of $^{14}CO_2$ from carboxyl-labelled histidine can occur in the absence of enzyme and pyridoxal phosphate but in the presence of an organic compound, AY-17,224. Finally, our studies using a boiled enzyme blank and the studies of Levine & Watts (1967) who used a potent inhibitor of histidine decarboxylase emphasize the fact that estimation of enzyme blanks by the omission of enzyme is not always satisfactory.

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The effect of hypoxia on isonicotinic acid hydrazide-induced seizures in chicks during ontogenesis

The concentration of γ-aminobutyric acid (GABA) in the brain of various species is elevated by hypobaric hypoxia, or by hypoxia caused by the breathing at ambient pressure of a gas mixture of low oxygen content (Wood, Watson & Ducker, 1968). Hypobaric hypoxia also delays the onset of semi-carbazide- and methionine sulphoximine-induced seizures (Baumel, Schatz & others, 1969) and it has been suggested by Baumel & others that the elevation in brain GABA level may be responsible for the anticonvulsant action. Although the above studies clearly indicate that both an elevation in brain GABA concentration and an anticonvulsant action are associated with hypoxic conditions, the results do not necessarily indicate a causal relation between the first two phenomena. The present investigation was therefore initiated to obtain additional information about this.

GABA metabolism increases during ontogenesis in the chick (Sisken, Sano & Roberts, 1961). If there is a concomitant change in the sensitivity of the brain GABA levels to hypoxia, this would allow a comparison to be made between the elevation of GABA and the anticonvulsant action of hypoxia in chicks of different ages. We have found that the sensitivity of brain GABA concentrations to hypoxia changes with age and that the elevation in brain GABA concentration is related to the delay in onset of the seizures.

White Leghorn cockerels aged 2 and 22-days were used. Isonicotinic acid hydrazide (INH) (2·18 mmol/kg) was injected intramuscularly in a volume of 0·154 M NaCl equivalent to 1% of body weight. The chick was then kept in a normal air environment or exposed immediately at ambient pressure to a hypoxic gas mixture containing 7·5% oxygen in nitrogen at 3–4 litre/min in a small chamber previously flushed with the mixture. The birds were observed continuously and the time to onset of generalized seizures recorded. The CT50 value was used as a quantitative measure of sensitivity. This value was the time required (min) after the administration of INH for seizures to occur in 50% of the birds. It was determined by plotting the percent convulsions on logarithmic probability paper (Miller & Tainter, 1944). Brain GABA levels were determined in control chicks (air) and in birds that had been exposed to hypoxic conditions for 20 min. No hydrazide was administered in these experiments. Brain extracts were prepared and the GABA assayed (Wood & Abrahams, 1971).

The CT50 values for the 2-day old chicks were 21·4 and 27·1 min for air and hypoxia respectively. For the 22-day old birds the values were 22·6 and 31·3 min. Hypoxic conditions delayed the onset of seizures in birds of both age groups, but the effect was more pronounced in the older birds, the changes in CT50 values being 26 and 38% in 2-day and 22-day old birds, respectively (Table 1).

Table 1. *Effect of hypoxia on brain GABA concentrations and on INH-induced seizures.*

Age (days)	Condition	GABA (μmol/g)	CT50† (min)	Change due to hypoxia (%) GABA	Change due to hypoxia (%) CT50	$\frac{\Delta CT50‡}{\Delta GABA}$
2	Normal	2·35 ± 0·03* (10)	21·4			
2	Hypoxic	2·56 ± 0·01 (5)	27·1	+ 9·0	+26·6	2·96
22	Normal	2·80 ± 0·05 (5)	22·6			
22	Hypoxic	3·17 ± 0·03 (5)	31·3	+13·2	+38·5	2·92

* Mean ±s.e.; values in parenthesis indicate number of samples per group.

‡ ration = (% change in CT50 due to hypoxia)/(% change in GABA concentration due to hypoxia).

† Time required after administration of INH for 50% of the chicks to convulse.

In agreement with Sisken & others (1961), the brain GABA concentrations increased during the first 3 weeks of life (Table 1). Moreover the change produced by a standard hypoxic condition was greater in the 22-day old birds than in the 2-day old chicks. The constant ratio (Table 1) indicates that the elevation in GABA is proportional to the anticonvulsant effect (i.e. change in CT50) thereby lending support to the hypothesis of Baumel & others that the elevation in brain GABA concentration is responsible for the anticonvulsant action of hypoxia. However, the evidence is still not unequivocal.

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Triglyceride and precursor concentrations in the fatty liver of rats after chronic administration of ethanol

The most widely held explanation of the induction of fatty liver by ethanol is that an increase in the $NADH_2$:NAD ratio, secondary to the oxidation of ethanol, favours the formation of α-glycerophosphate and impairs the oxidation of fatty acyl-CoA derivatives (Lieber, Rubin & de Carli, 1969). The resulting increase in the concentrations of both precursors should then favour esterification to form triglyceride. In apparent support of this view, a single dose of ethanol has been found to increase the hepatic concentration of α-glycerophosphate and of triglycerides (Nikkilä & Ojala, 1963; Zakim, 1965). We have examined the concentrations of both precursors and product after chronic administration of ethanol in doses which consistently give rise to fatty liver in the rat.

Male Wistar rats, 200–300 g, were given homogenized liquid diets (Khanna, Kalant & Bustos, 1967) for 14 and 21 days. One diet, freely available, provided 35% of the total calories as ethanol, 19% as protein hydrolysate, 5% as sucrose and 41% as fat, and was nutritionally adequate in all other respects. The daily intake of ethanol averaged 10–12 g/kg. In a second diet the pair-fed controls had ethanol replaced by a calorically equivalent concentration of sucrose.

At the end of the treatment the animals were decapitated, the abdomen opened, and a portion of liver frozen instantly *in situ* by Wollenberger tongs precooled in liquid nitrogen. The frozen tissue was ground, deproteinized in 6% (w/v) perchloric acid, and centrifuged at 20 000 *g* for 15 min. The precipitate contained the long-chain fatty acyl-CoA derivatives, which were hydrolysed (Bortz & Lynen, 1963) and assayed for CoA content (Stadtman, 1955). α-Glycerophosphate was measured enzymatically in the neutralized supernatant (Hohorst, 1963). Other portions of the same livers were homogenized in phosphate buffer, and the triglycerides were extracted and measured (Butler, Maling & others, 1961).

Table 1. *Hepatic concentrations of triglycerides, long-chain acyl-CoA derivatives and α-glycerophosphate in rats consuming isocaloric ethanol and sucrose liquid diets.* Values shown represent mean ± s.e.

Time (days)	Group	Triglycerides (mg/g liver, wet weight)	Long-chain Acyl-CoA (n mol/g liver, wet weight)	α-Glycerophosphate (n mol/g liver, wet weight)
14	Ethanol	52·8±2·5 (4)‡	20·50±2·10 (9)‡	1347±124 (9)‡
	Sucrose	15·2±0·86* (4)	18·80±2·20 (9)	1210±58 (9)
21	Ethanol	64·6±1·8 (4)	27·80±3·50 (5)	943±149 (5)
	Sucrose	17·3±1·2* (4)	18·80±3·0† (5)	1195± 59 (5)

* Differs from ethanol group ($P < 0.01$). † Differs from ethanol group ($P < 0.02$).
‡ No. of animals.

An increase in liver triglyceride content in the ethanol groups was found at 14 and 21 days, compared with their respective pair-fed controls (Table 1). The concentration in the 21 day ethanol group was significantly higher than in the 14-day group ($P<0.01$). However, no significant difference was found in α-glycerophosphate levels at either time. The concentration of long-chain fatty acyl-CoA derivatives was increased in the ethanol group only at 21 days, but not at 14 days.

The results do not support the hypothesis that fat accumulation in this situation results from a rise in esterification owing to increase in the concentrations of the precursors. The concentration of long-chain fatty acyl-CoA derivatives was increased at 3 weeks, but this change followed the development of fatty liver, rather than preceding it. Since the secretion of hepatic lipoproteins into the plasma is increased rather than decreased by ethanol (Baraona & Lieber, 1970) three alternative possibilities deserve consideration. (1) Production and turnover of the precursors may be increased during ethanol oxidation, but the equilibrium constant of the esterification reaction is far over to the side of the triglyceride, so that the reaction velocity is not markedly influenced by precursor concentrations. (2) The increased triglyceride concentration does not represent the pool of newly synthesized product, but a sequestered compartment (e.g., cytoplasmic fat droplets), the size of which is independent of the rate of esterification. (3) Both of the above are true.

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Angiotensin tachyphylaxis and the uptake of [^{14}C]angiotensin in guinea-pig aortic strips

The development of tachyphylaxis in response to angiotensin in guinea-pig isolated aortic strips has been attributed to a change in the receptor mechanism(s) for angiotensin (Palaic & LeMorvan, 1971). We now report the correlation of the change in reactivity to angiotensin and the uptake of [^{14}C]angiotensin in aortic strips.

The thoracic aorta from male guinea-pigs, 250–300 g, was cut spirally in a strip approximately 6 mm wide and 3 cm long. This was divided into two equal parts by longitudinal dissection to give two matching strips from one animal. The strips were mounted in 10 ml baths in Krebs solution at 37°, with 10^{-5}M of EDTA and allowed to relax for 2 h. One strip had drugs, while the other served as the control. The isotonic contractions were recorded on a Physiograph (E & M Co.).

Only concentrations of angiotensin giving maximal responses (around 10^{-7}M and more) were capable of producing tachyphylaxis (Palaic & LeMorvan, 1971). Because of the heterogeneity of receptors in aortic strips, it is impossible to estimate the contractile response to one agonist on the basis of the contractile response to another (Altura & Altura, 1970). Therefore we used only successive doses of the same concentration of angiotensin to produce tachyphylaxis.

The degree of tachyphylaxis achieved after successive maximal doses of angiotensin increased with duration of contact between the angiotensin and the aortic preparation and with decreased recovery time after each contraction, but recovery time could not be shortened to less than 10–12 min, that being the minimum time required for the strip to relax to the base line. A 3 min contact with the agonist and 12 min recovery time, was therefore employed throughout. The time required to achieve a 50% decrease from the initial maximal response (DMR50) may serve as a reliable index of tachyphylaxis. DMR50 values for different concentrations of angiotensin were plotted and the straight line obtained showed tachyphylaxis to be directly proportional to the concentration of angiotensin. The theoretical implications that may possibly be considered from such a relation could have a bearing on the change in receptor reactivity with respect to time and the relative number of receptors involved. One could also postulate the existence of "negative" tachyphylaxis for submaximal doses, this in fact being an autostimulation or autopotentiation. The phenomenon has been already described by Godfraind (1968) and we have observed it with submaximal doses of angiotensin in guinea-pig aorta. The nature of this interesting phenomenon is unknown.

Table 1. *Uptake of ^{14}C-angiotensin by guinea-pig aortic strips and vas deferens.* Aortic strips were made tachyphylactic to 10^{-6}M angiotensin and then incubated with 1·6 μg/ml of [^{14}C] angiotensin (125 nCi/ml) at 37° for 15 min. Results expressed as d min^{-1} mg^{-1} of dry tissue; individual results. Statistical analysis was made using the paired *t*-test.

Aorta		Vas deferens
Control	Tachyphylactic	
759	882	185
682	911	216
771	877	202
941	967	185
1090	1188	221
1234	1470	
912 ± 88·0	1049 ± 96·6	202 ± 20·9
	$P < 0{\cdot}01$	

A major, as yet unresolved problem in the pharmacology of smooth muscle is how to correlate the contractile response with the binding capacity of muscle for an agonist. It was thought that a smooth muscle preparation in which a change in reactivity, such as tachyphylaxis, is produced by the agonist itself might be a useful model for such a study. In our experiments shown in Table 1, aortic strips were firstly made tachyphylactic to 10^{-6}M angiotensin. They were then transferred to a beaker containing 1·6 μg/ml of [^{14}C]angiotensin (125 nCi/ml) in 2 ml of Krebs solution, and incubated in a Dubnoff shaker at 37° for 15 min. The strips were subsequently washed and prepared for counting in a Packard liquid scintillation counter. A vas deferens taken from the same animal was incubated and counted in the same fashion as the aortic strips. Since the vas deferens does not respond to angiotensin, it was considered as a smooth muscle without specific receptors for angiotensin. The results are expressed as d min^{-1} mg^{-1} of dry tissue. Statistical analysis was made using the paired *t*-test.

Our results show that the uptake of [^{14}C]angiotensin is significantly higher in tachyphylactic strips than in the controls ($P<0{\cdot}01$). This was not due to the change in extracellular space, since the inulin space was the same in tachyphylactic strips as in the controls. The amount of [^{14}C]angiotensin bound to the vas deferens was only one quarter of that found in aortic strips, indicating the possible degree of non-specific binding to the smooth muscle.

The increased binding of [^{14}C]angiotensin indicates that the number of receptive sites is increased during the development of tachyphylaxis. This might be due to the induction of new receptors, either by *de novo* synthesis or by conformational changes of the existing receptor proteins.

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Biliary excretion of [^{14}C]edrophonium and its glucuronide conjugate

Although edrophonium chloride (ethyldimethyl(3-hydroxyphenyl)ammonium chloride) is occasionally used for diagnostic purposes in man, little is known of its metabolism and excretion. In general, chemically reversible anticholinesterase drugs have rapid and evanescent effects (Randall, 1950); in these conditions, detoxication may have little or no influence on pharmacological action. Indeed, it is commonly assumed that most quaternary amines are not metabolized, since the lipophilic endoplasmic reticulum may restrict the penetration and limit the metabolism of polar compounds (Gaudette & Brodie, 1959). These limitations do not necessarily apply to all polar drugs (Mazel & Henderson, 1965) or low molecular weight quaternary compounds (Somani, Wright & Calvey, 1970). For these reasons, and since no previous studies of the metabolism of edrophonium have been reported, we have studied the excretion of this drug and its metabolic products in bile.

The common bile duct of Wistar rats of either sex was cannulated under urethane anaesthesia, and bile was collected at hourly intervals after intravenous injection of [^{14}C]edrophonium chloride (2·0 μmol/kg) in saline. Specimens were assayed for radioactivity by liquid scintillation spectrometry, and [^{14}C]edrophonium and its metabolites were detected by paper and thin-layer chromatography in at least five different solvent systems. Conjugates of edrophonium in bile were identified by incubation with β-glucuronidase; control specimens were incubated with the enzyme in the presence of the specific inhibitor glucaro-(1→4)-lactone.

In 24 experiments, 4·7 $\pm$ 2·3% (mean $\pm$ s.d.) of the dose of [^{14}C]edrophonium was excreted in bile in 6 h. The only significant metabolite identified was a 3-oxyglucuronide conjugate of edrophonium {[^{14}C]ethyldimethyl(3-oxyphenyl)ammonium glucuronide}. Approximately 89% of the radioactivity in bile was present as this metabolite 1 h after administration of the drug (Table 1); some 10% was excreted as [^{14}C]-edrophonium. Only small amounts of the unchanged drug were identified 2–6 h after injection. Thus, the presence of unchanged edrophonium in bile may represent diffusion of the drug from periductular plasma downstream from the biliary canaliculus.

The trimethyl analogue of edrophonium (trimophonium) is partly demethylated before biliary excretion (Somani & others, 1970). In the present experiments, approximately 1% of the total radioactivity in bile was eliminated as an unidentified metabolite; it is possible that this substance represents a dealkylated conjugate of edrophonium.

Table 1. *Excretion of* [^{14}C]*edrophonium and its glucuronide conjugate in normal rat bile.* Values (based on mean $\pm$ standard deviation of at least five experiments) represent the proportion of total radioactivity present as each compound.

Time (h)	Proportion of total radioactivity (%) [^{14}C]Edrophonium	[^{14}C]Edrophonium glucuronide
1	10·4 $\pm$ 3·8	88·9 $\pm$ 3·8
2	1·3 $\pm$ 0·5	97·9 $\pm$ 0·8
3	0·9 $\pm$ 0·3	98·2 $\pm$ 0·4
4	1·0 $\pm$ 0·3	98·2 $\pm$ 0·3
5	0·7 $\pm$ 0·1	98·2 $\pm$ 0·4
6	0·7 $\pm$ 0·3	97·8 $\pm$ 0·6

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A common error in assessing the significance of percentage change in neuropharmacology

It has become common practice to express values for drug-treated animals as a percentage of (or as a percentage change from) values observed in saline controls. This procedure would be straightforward and easily understood if one could measure the effect of drugs in each experimental animal both after a saline control treatment and after a drug treatment, or if there existed some rational justification for pairing individual saline and drug-treated animals and if the numbers of animals employed in each treatment were the same. However, the calculation and interpretation of percentages is not so simple when these requirements are not met.

We have noted that the standard errors of mean percentages published in many experiments of this kind are erroneously small. In these instances, the standard error is unjustifiably small because it reflects only the variation within the drug sample and does not reflect the variation inherent within the control sample.

The error arises in one of two ways. It is made in one way when the standard error of the mean percentage change is calculated from a series of percentages, each derived from an observation made on an individual drug-treated animal, by dividing the mean percentage change by the mean of the controls. It is made in another way when the standard error of the mean percentage change is obtained by dividing the standard error of the drug mean by the mean of the controls.

Let us consider a hypothetical experiment involving a certain drug and brain 5-hydroxytryptamine concentrations. We assume that this drug has no effect on the concentration of 5-HT and hence that the experimental animals should have the same average value for 5-HT as do the saline controls. Let us assume that the available animals are from a normal population having a 5-HT concentration with a mean and standard deviation of 1000 and 150 ng/g brain. Thus, if one randomly takes 25 animals for each of the two groups the standard deviation of the sample mean of the control as well as the treatment group would be $150/\sqrt{25} = 30$ ng/g. Thus, it would be quite reasonable to obtain sample means of 1045 and 965 ng/g 5-HT for the saline and drug group, respectively. If one ignores the variability of the control group we can estimate that the figure for the drug group is 92·3% of that of the control group with a standard error of $(30 \times 100)/1045$ or 2·87%. We then see that the drug appears to lower brain 5-HT by 7·7% which is 2·68 standard errors lower than the control and normally the conclusion would be made that the drug significantly lowers brain 5-HT. However, if the variability in the control group is considered, the standard error for the ratio of the two means, say, $\bar{X}/\bar{Y}$, must be

obtained. An estimate (Duncan, 1965) of this standard error is given by:

$$\sigma_{\bar{X}/\bar{Y}} = ((\bar{Y}^2\sigma^2_{\bar{X}} + \bar{X}^2\sigma^2_{\bar{Y}})/\bar{Y}^4)^{1/2} \quad .. \quad .. \quad .. \quad .. \quad (1)$$
$$= [(1045^2 \times 900 + 965^2 \times 900)/1045^4]^{1/2}$$
$$= 0{\cdot}039$$

or on a percentage basis this would be 3·9%. Thus, the decrease of 7·7 would only be 1·93 standard errors and hence would not be as readily accepted as statistically significant.

When nine animals are used for the control group and 25 for the drug group the results are even more startling. The standard error of the saline group would be 50 ng/g, and a sample mean of 1060 would not be unreasonable. The former method of calculation would produce a percentage decrease of 8·96% compared to the same standard error of 2·87% which is 3·12 standard errors (a highly significant value). The standard error of the ratio $\bar{X}/\bar{Y}$, however, is:

$$\sigma_{\bar{X}/\bar{Y}} = [(1060^2 \times 900 + 965^2 \times 2500)/1060^4]^{1/2}$$
$$= 0{\cdot}048$$

or 4·8%. Thus, the decrease of 8·96% is only 1·87 standard errors compared to the 3·12 standard errors obtained above.

The consequence of this kind of error is also important in the comparison of the relative effect of a drug upon substances in the brains of animals arising from two different prior treatments. The example presented here will exhibit the situation where the saline control groups for the two treatment conditions are significantly different. It should be evident in this circumstance that absolute drug-induced changes could differ significantly although the proportional changes might not be significantly different, or *vice versa*. Thus, the formulation of the hypothesis before experimentation becomes important.

In such cases, one popular erroneous method of testing for significant differences in the effect of a drug for different prior treatments involves: (i) expressing the

Table 1. *Three methods for testing for significant differences of drug-induced changes.*

	Prior treatment 1		Prior treatment 2	
	Saline (X)	Drug (Y)	Saline (X)	Drug (Y)
Number of samples	30	30	30	30
5-HT (ng/g), Mean and standard error	702 ± 19·4	794 ± 22·0	913 ± 25·3	935 ± 25·9
Drug-induced changes (Δ)				
Method				
I Standard error of Δ obtained by $\left(\sigma^2_{\bar{X}} + \sigma^2_{\bar{Y}}\right)^{1/2}$.	92 ± 29·3		22 ± 36·2	
Test of difference in Δ's		70 ± 46·6 t = 1·50		
II Standard error of Δ percentage obtained by equation (1)	13·10 ± 4·49		2·40 ± 4·01	
Test on difference in Δ's		10·70 ± 6·02 t = 1·78		
III Standard error of Δ percentage obtained by $\left(\sigma_{\bar{X}}\right)/\bar{Y}$	13·10 ± 3·13		2·40 ± 2·83	
Test on difference in Δ's		10·70 ± 4·22 t = 2·54		

Table 2. *Analysis of variance table for 5-HT measurements in ng/g.*

Source of variation	Degrees of freedom	Sums of squares	Mean square	*F*	$\sqrt{F}$
Prior treatments (T) ..	1	929 808	929 808		
Control vs. drug (d) ..	1	97 527	97 527		
T D interaction ..	1	36 855	36 855	2·27	1·51
Error	116	1 885 476	16 254		
	119	2 949 666			

Table 3. *Analysis of variance table for the logarithm of the measurement.*

Source of variation	Degrees of freedom	Sums of squares	Mean square	*F*	$\sqrt{F}$
Prior treatments (T) ..	1	0·259 777	0·259 777		
Control vs. drug (D) ..	1	0·031 360	3·031 360		
T D interaction ..	1	0·014 531	0·014 531	3·22	1·80
Error	116	0·522 801	0·004 507		
	119	0·828 469			

standard error of drug-treated animals for each prior treatment as a percentage of the mean saline control for that prior treatment, and (ii) using the "standard errors" thus derived in a *t*-test to compare the percentage drug-induced change for the different prior treatments. It is the standard error of these differences that is in error. Table 1 summarizes a set of figures analysed for significance, first by the actual measurements in ng/g, second by the use of equation (1), and third by using actual measurements and testing for percentage differences by ignoring the uncertainty in the saline control groups. Tables 2 and 3, respectively, were constructed by making an analysis of variance on the absolute measurement data and on the logarithm of each measurement. The first test in Table 1 is equivalent to the test on the interaction term in Table 2. It should be recalled that the F distribution with 1 and n degrees of freedom is equal to the square of the *t* distribution with n degrees of freedom. By comparing the results of the second test in Table 1 with the interaction test in Table 3 one sees that the analysis of variance using logarithms is equivalent to the test for significant percentage changes. The third test in Table 1 ignores the variability in the saline control samples and therefore gives the erroneous impression that animals experiencing the two prior treatments respond differently to the drug.

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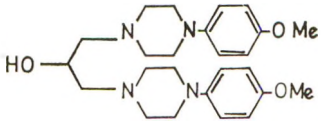
1,3-Bis[4-(*p*-methoxyphenyl)piperazinyl]-2-propanol (Ro 8-2580): a new monoamine depletor

Dopamine, noradrenaline and 5-hydroxytryptamine (5-HT) are depleted by reserpine, benzoquinolizine derivatives, oxypertine (Bak, Hassler & Kim, 1969; Carlsson, Jonasson & Rosengren, 1963), prenylamine and ϵ-aminocaproic acid (for review see Glowinski, 1970; Haefely, 1968) and, more recently, U-20057 (Johnson & Rudzik, 1970).

The present paper describes a new compound that decreases monoamines and produces slight sedation: 1,3-bis[4-(*p*-methoxyphenyl)-1-piperazinyl]-2-propanol, mol.wt 440·6 (Ro 8-2580).*



Rats from a closed randomized colony (Füllinsdorf strain, 80–160 g) were given single doses of Ro 8-2580 by stomach tube.

For amine determinations the animals were decapitated and the brains and hearts homogenized, extracted (Shore & Olin, 1958) and the content of noradrenaline (modification of Shore & Olin, 1958), dopamine (Bertler, Carlsson & Rosengren, 1958), 5-HT (Bogdanski, Pletscher & others, 1956) and 5-hydroxyindolacetic acid (5-HIAA) (Udenfriend, Weissbach & Brodie, 1958) measured.

For pharmacological investigations the following tests were made in separate animals.

The "openfield" test (Janssen, Jagenau & Schellekens, 1960): the number of "walking" and "rearing" movements (exploratory activity) were noted for 3 min. The catalepsy test (Boissier & Simon, 1963): the animal was considered to be cataleptic if the crossed homolateral limbs remained in this unnatural position for at least 10 s. Prolongation of pentobarbitone sleeping time: the number of animals losing their righting reflex for more than 1 min, 30 min after a intraperitoneal subhypnotic dose of pentobarbitone (12·5 mg/kg) was noted. The locomotor activity of two groups of three rats was measured in activity cages (Lehigh-Valley, Electronics Inc., Mod. A 2497) simultaneously with two control groups; the light beams interruptions were counted every 5 min. The performance of rats on a rotating rod was examined as described by Dunham & Mija (1957). The rectal temperature was obtained by means of a telethermometer equipped with a No. 402 probe (Yellow Springs Instrument Co.).

All three brain amines are below 50% of controls between 2 and 4 h after 100 μmol/kg Ro 8-2580 (Fig. 1); the 5-HT is the least affected (60% below) and dopamine the most (90% below). The 5-HIAA content increased (90% above) as the 5-HT decreased.

The Ro 8-2580-induced decline of 5-HT in brain (60% at 2 h) differs from that of noradrenaline (75%) in brain and heart at 2 and 8 h respectively). The recovery of 5-HT proceeded at a higher rate than that of noradrenaline, regaining its normal brain concentration after about 24 h, whereas the catecholamines, especially noradrenaline in the heart, were only restored at 48 h or even later. The relatively rapid decrease of the brain amines is paralleled by a diminished exploratory activity (rearing

* Synthetized by Dr. A. Edenhofer, Chemical Department, F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland.

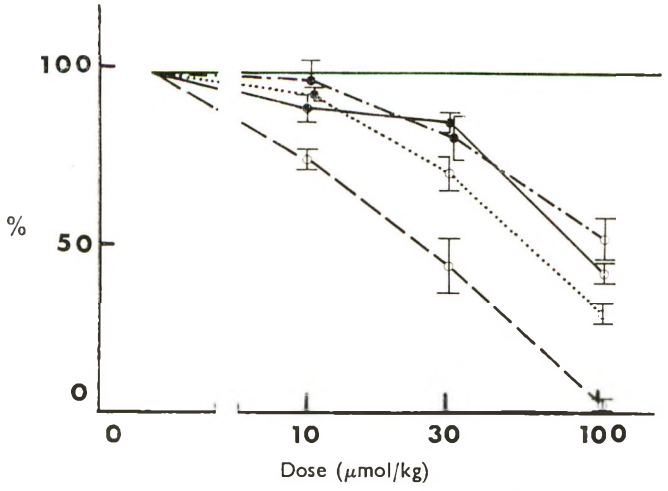


FIG. 1. Decrease of monoamines 2 h after oral application of increasing doses of Ro 8-2580. At time 0, the dose of the depletor was given by mouth. Control animals was taken as 100% (ordinate). Each point represents a mean value of at least three experiments ± standard error. Open circles are significantly different ($P < 0.01$) from controls. — 5-HT, — — dopamine, ··· noradrenaline in brain. —·— Noradrenaline in heart.

and walking); the recovery of the activity after 24 h was quicker than that of the amines. In spite of this difference it appears that motor activity in an unfamiliar environment (exploratory activity) correlates much better with the amine depletion than does the motor activity in a familiar environment (motor activity) which showed a significant diminution during about 3 h only. Decreased performance in the rotarod test had about the same time course. Unexpectedly, Ro 8-2580 neither produced catalepsy nor potentiated pentobarbitone sleeping time nor decreased the temperature.

Interference with the storage capacity constitutes the most likely mechanism for the monoamine lowering effect of Ro 8-2580. The concomitant increase in 5-hydroxyindolacetic acid suggests that the decrease of amine content is not due to inhibition of synthesis but to a depleting action. Ro 8-2580 has about the same duration of action as the benzoquinolizines (e.g. Ro 4-1284) (Pletscher, Brossi & Gey, 1962), U-20057 (Johnson & Rudzik, 1970) and oxypertine (Bak & others, 1969) and therefore differs from the longer acting reserpine. The depletion of catecholamines is more pronounced than that of 5-HT, as also reported for benzoquinolizines (Pletscher & others, 1962).

Many investigators have reported (e.g. Pirch, 1969; Pirch, Rech & Moore, 1967; Faith, Young & others, 1968) that the sedative action of reserpine rapidly recovers within 48 h, while amine content remains low. These observations suggest a dissociation of brain amine content and sedation (Brodie & Costa, 1962; Carlsson & others, 1963). The results of the present study confirm such observations with a new amine depletor.

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