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A quantitative analysis of the antagonism of intravenous noradrenaline by thymoxamine or phentolamine on the blood pressure of the conscious cat

A. T. BIRMINGHAM, P. I. AKUBUE* AND J. SZOLCSÁNYI†

The blood pressure of the unanaesthetized cat was recorded from a nylon catheter inserted permanently into the aorta via the right carotid artery and intravenous injections were made into a nylon catheter tied permanently into the right external jugular vein. For noradrenaline there was a linear relation between log dose and blood pressure rise. The α-blocking agents thymoxamine and phentolamine lowered the blood pressure and decreased the pressor response to noradrenaline. Quantitative analysis of the results by three different graphical methods revealed that for the first dose of blocking agent the antagonism was complex but had the characteristics associated with mixed non-competitive and uncompetitive antagonism. For further cumulative doses of blocking agent, the antagonism had the characteristics of competitive antagonism. It is concluded that a first dose of α-blocking agent has an effect on the dynamics of a noradrenaline-induced blood pressure rise in the conscious cat which is complex and could in part be due to the initial effect of the blocking agent in lowering the blood pressure and in part to the activity of the vasomotor compensatory reflexes. After α-blockade has been initiated, the characteristics of the antagonism are, like those in the anaesthetized cat, those of competitive antagonism.

QUANTITATIVE analysis of the effects of adrenergic blocking agents on the responses of the cardiovascular system to injected catecholamines is likely to be more complex than that for adrenergic blockade of vascular tissue *in vitro*, but Chen & Russell (1950) and Matsumoto & Kumoi (1958) showed, for anaesthetized animals, that by suitable graphical treatment of the results such analysis was possible. Thuránszky (1966a) established that there are qualitative differences between anaesthetized and unanaesthetized cats in the responses of the blood pressure to adrenaline and to adrenergic blocking agents.

This paper describes a quantitative analysis of the blood pressure responses of unanaesthetized cats to intravenous noradrenaline alone and to noradrenaline in the presence of adrenergic blocking agents. For one group of cats the α-blocking agent was thymoxamine, 4-(2-dimethylaminoethyl)-5-isopropyl-2-methylphenyl acetate, which was shown by Birmingham & Szolcsányi (1965) to be a competitive antagonist of noradrenaline on isolated arterial strips and by Greef & Schümann (1953) to reduce or abolish in anaesthetized cats, the rise in blood pressure associated with the intravenous injection of noradrenaline. For a second group of cats the α-blocking agent was phentolamine (Meier & Yonkman, 1949; Walker, Heymans, Wilson & Richardson, 1950).

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Experimental

METHODS OF CANNULATION

Blood pressure was recorded from a catheter placed permanently in the aorta and drugs were injected through a catheter tied permanently into the external jugular vein. The method of cannulation and daily management of the cats was based on that developed by Thuránszky (1966b). Cats of either sex weighing from 1·75 to 3·5 kg were anaesthetized with ether. The right external jugular vein was exposed through an incision to the right of the midline and cannulated with Portex No. 3 standard nylon tubing of outside diameter 1·25 mm and bore 0·75 mm. Some 3 cm of tubing was inserted into the vein and about 7 cm protruded. The external end was pushed into closely fitting soft rubber tubing, about 3 cm in length, and sealed with a glass stopper after filling with saline. The catheter was tied firmly into the vein by three ligatures. Next, the right carotid artery was prepared for cannulation and at this stage 0·1 ml of 5,000 IU/ml heparin was injected into the venous cannula. The arterial cannula (also Portex No. 3) was approximately the same length as the distance from the xiphoid process to the tip of the mandible. The catheter was connected by a soft rubber tube to a syringe, filled with saline and inserted into the carotid artery for about 2 cm and held in place by one loosely-tied ligature. The syringe was now replaced by a polythene tube leading to a Statham pressure transducer, and while the blood pressure was recorded, the arterial catheter was advanced into the aorta until about 8 cm remained. At this point, with the tip of the catheter in the descending thoracic or upper abdominal aorta, two firm ligatures were tied around the catheter in the carotid artery. The catheter was then filled with heparin and closed with a glass stopper. The incision was closed with Michel clips leaving both catheters protruding from the top end. The incision and the catheters were protected with a cotton bandage. Although the cats made a quick recovery from the operation they were not used in an experiment until the fourth day because Thuránszky, Rablóczky & Kékes-Szabó (1966) showed that the responses to injected catecholamines were altered by anaesthesia and this alteration persisted, in varying degrees, for 72 hr after the recovery of consciousness.

EXPERIMENTS

During an experiment each cat was housed in a large cage within which it could move freely. The bandage was removed and the venous cannula was connected to a long polythene tube through which drug solutions were injected. The dead space of the system was 1·4 ml. The arterial cannula was similarly connected to a Statham pressure transducer for recording blood pressure on a Grass 7 Polygraph. Both polythene tubes were elastically-suspended above and through the roof of the cage so that the movements of the cat were not impeded. The experiment was not begun until the cat was lying at rest and the blood pressure had stabilized. After ensuring that there was no change in blood pressure to injections of

normal saline, the responses to intravenous noradrenaline were recorded. Doses of 0·1, 0·2, 0·4 and 0·8 μg/kg noradrenaline were injected with an interval of 4 to 5 min between doses. Then the first dose of adrenergic blocking agent, thymoxamine or phentolamine, was injected. When the blood pressure had stabilized again the same four doses of noradrenaline were repeated. This procedure was repeated until a total of three doses of adrenergic blocking agent had been given. The cats showed no signs of disturbance from the changes in blood pressure induced by the drugs. Different cats were used for thymoxamine and for phentolamine. For the few cats used more than once, at least 3 days were allowed to elapse between experiments.

DRUGS

These were: noradrenaline bitartrate, phentolamine hydrochloride and thymoxamine hydrochloride; they were dissolved in normal saline and doses refer to the base/kg bodyweight.

Results

ANTAGONISM OF NORADRENALINE BY THYMOXAMINE OR PHENTOLAMINE

Table 1 shows the mean results for thymoxamine obtained from six experiments made on five cats. Before thymoxamine, the noradrenaline-induced rise in blood pressure increased with each increase in dose (x) of noradrenaline, the increase in blood pressure (y) ranged from 30 mm Hg for 0·1 μg/kg noradrenaline to 90 mm Hg for 0·8 μg/kg. After treatment with 4 mg/kg thymoxamine, the same doses of noradrenaline produced much smaller rises in blood pressure (y′). Two more increments of 4 mg/kg thymoxamine produced further but smaller reductions in the responses to the four doses of noradrenaline. Also shown in Table 1 are the mean results for phentolamine from seven experiments on another five cats.

TABLE 1. MEANS OF THE PRESSOR RESPONSES TO INTRAVENOUS NORADRENALINE ALONE AND IN THE PRESENCE OF THYMOXAMINE OR PHENTOLAMINE (the standard errors are shown in parentheses)

x, the dose of noradrenaline (μg/kg)	y, the pressor response to noradrenaline (mm Hg)	y′, the pressor response to noradrenaline (mm Hg) after each cumulative dose of antagonist		
		Thymoxamine mg/kg		
		4	8	12
0·1	30·2 (3·7)	20·7 (2·3)	16·0 (3·5)	14·5 (5·3)
0·2	52·8 (4·5)	34·2 (4·4)	25·8 (2·9)	24·8 (5·1)
0·4	72·0 (8·0)	45·3 (5·1)	44·3 (8·1)	37·5 (7·8)
0·8	90·5 (9·8)	55·5 (5·3)	54·5 (8·1)	49·0 (9·6)
		Phentolamine mg/kg		
		0·2	0·4	0·6
0·1	18·6 (2·1)	12·4 (1·3)	10·0 (2·2)	6·0 (4·0)
0·2	38·1 (4·5)	25·0 (5·2)	16·9 (4·4)	16·2 (5·4)
0·4	56·1 (5·0)	37·8 (7·6)	33·4 (5·0)	32·0 (7·5)
0·8	81·3 (9·6)	51·6 (7·5)	50·9 (6·4)	42·7 (8·1)

The results from Table 1 are shown as log dose response graphs, with regression lines, in Fig. 1. The logarithm of the noradrenaline dose

(log x) was plotted as abscissa against the rise in blood pressure as ordinate for noradrenaline alone (y) and for noradrenaline after the first, second and third dose of antagonist (y'). For noradrenaline alone there was a linear relation between log dose and response. After 4 mg/kg thymoxamine or 0·2 mg/kg phentolamine the relation was still linear but the response to noradrenaline was reduced. The reduction was greater the higher the noradrenaline dose so that the regression line had a decreased slope. After each subsequent dose of 4 mg/kg thymoxamine or 0·2 mg/kg phentolamine there was a further decrease in the response to noradrenaline. The reductions were less pronounced than that produced by the first dose of antagonist and there appeared to be no further change in the slopes of the regression lines.

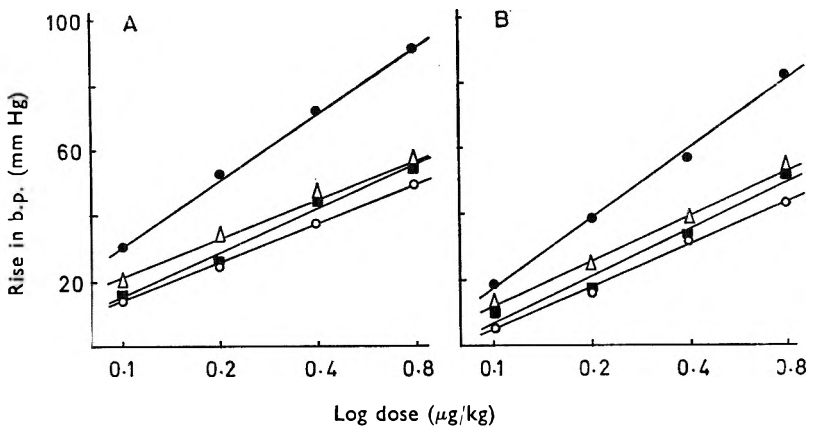

FIG. 1. Log dose-response graphs of the effects of thymoxamine (A) or of phentolamine (B) on the rise in blood pressure induced by intravenous noradrenaline in the conscious cat. The ordinates show the increase in blood pressure in mm Hg (the blood pressure before the injection subtracted from the blood pressure at the height of the response to the noradrenaline injection). The abscissae show the doses of noradrenaline in μg/kg plotted on a logarithmic scale. A. Each point is a mean of six observations on five cats: ●——● the response to noradrenaline alone, △——△, ■——■ and ○——○ the responses to noradrenaline after cumulative total doses of 4, 8, and 12 mg/kg thymoxamine respectively. B. Each point is a mean of seven observations on five cats: ●——● the response to noradrenaline alone, △——△, ■——■ and ○——○ the responses to noradrenaline after cumulative total doses of 0·2, 0·4 and 0·6 mg/kg phentolamine respectively.

In Fig. 2 the results from Table 1 are shown plotted as reciprocals of noradrenaline dose (abscissa), 1/x, against reciprocals of rise in blood pressure (ordinate) for noradrenaline alone, 1/y, and for noradrenaline in the presence of the antagonists, 1/y'. Calculated regression lines were fitted to each set of mean results. For noradrenaline alone there was a linear relation between 1/x and 1/y. After 4 mg/kg thymoxamine or 0·2 mg/kg phentolamine there was still a linear relation between 1/x and 1/y' but the lines had steeper slopes and different intercepts with the ordinates when compared with the control lines (1/x against 1/y). The subsequent doses of thymoxamine or phentolamine produced 1/x against

1/y′ plots each with an increased slope but all the lines in the presence of the antagonists had a common intercept on the ordinate. When the total dose of phentolamine had reached 0·6 mg/kg, the straight line relation appeared to break down at the lowest dose of noradrenaline as shown by the sharp increase in slope of the line joining the calculated line for the three higher doses to the point for the lowest dose of noradrenaline.

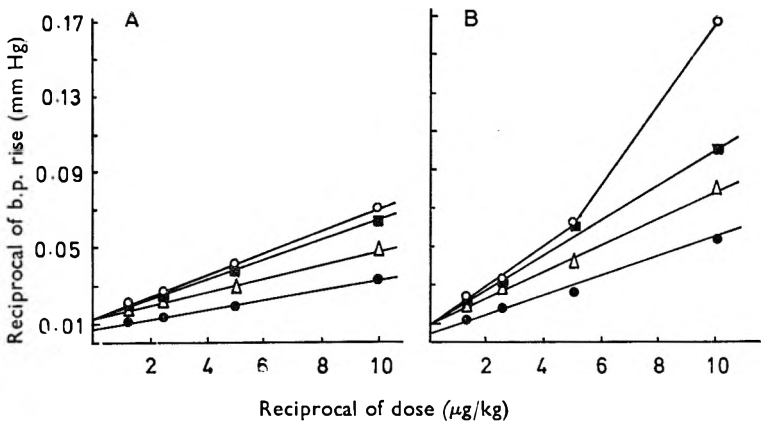

FIG. 2. Lineweaver and Burk plots of the results shown in Fig. 1. A. Thymoxamine. B. Phentolamine. Ordinates: the reciprocal of the increase in blood pressure in mm Hg. Abscissae: the reciprocal of the dose of noradrenaline in μg/kg injected intravenously. Key to symbols as in Fig. 1.

Further analysis of the antagonism of noradrenaline was made by plotting the logarithm of the dose of noradrenaline (log x) as abscissa against the logarithm of the rise in blood pressure (y or y′) divided by the difference between the rise in blood pressure and the maximum rise in blood pressure (y_{max} or y'_{max}) as ordinate (Fig. 3). The maximum rise in blood pressure was not measured directly but was calculated (Chen & Russell, 1950) from the point of intercept of the dose response line with the ordinate in Fig. 2. This analysis was made only for noradrenaline alone and for the first dose of phentolamine or thymoxamine. Regression lines were fitted to the points and Fig. 3 shows that there was a linear relation for log x against log $[y/(y_{max} - y)]$ or log $[y'/(y'_{max} - y')]$ when plotted by this method and that the lines for response to noradrenaline after one dose of antagonist were above and parallel to the lines for noradrenaline alone ($P > 0{\cdot}9$ for phentolamine; $P = 0{\cdot}8$ to $0{\cdot}9$ for thymoxamine).

THE EFFECTS OF THYMOXAMINE OR PHENTOLAMINE ON THE BLOOD PRESSURE

When thymoxamine (4 mg/kg) was injected there was usually a sudden and profound fall in blood pressure which lasted for 30–40 sec (approximately the period of injection) which was then replaced by a short-lived rise before the blood pressure stabilized at a level below that of the pre-injection pressure. There was no comparable sudden fall in blood

pressure with phentolamine, the injections of 0·2 mg/kg were usually followed by a slow and gradual fall in blood pressure to a new stable level below that of the pre-injection pressure. From the records made in the experiments on antagonism of noradrenaline, the effects of thymoxamine or phentolamine on the blood pressure of the conscious cat were measured as the difference between the stable pre-injection blood pressure

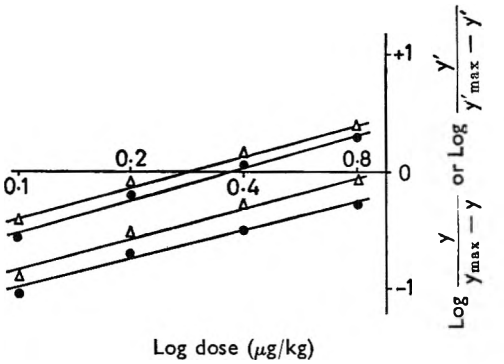

FIG. 3. The effects of the first dose of thymoxamine or phentolamine on the blood pressure responses to intravenous noradrenaline in the conscious cat. Ordinate: the logarithm of the rise in blood pressure divided by the difference between the observed rise and the calculated maximum rise. Abscissa: the doses of intravenous noradrenaline in μg/kg plotted on a logarithmic scale. *Upper two lines:* ●——● noradrenaline alone, △——△ noradrenaline after 4 μg/kg thymoxamine. Each point is the mean of six observations on five cats. *Lower two lines:* ●——● noradrenaline alone, △——△ noradrenaline after 0·2 μg/kg phentolamine. Each point is the mean of seven observations on five cats.

and the blood pressure at a point when it had again stabilized after the injection (usually 5–8 min post-injection). The means of these differences, plotted as cumulative fall in blood pressure in mm Hg, against log cumulative dose of antagonist are shown with regression lines in Fig. 4. For both thymoxamine and phentolamine there was a linear relation between fall in blood pressure and the logarithm of the cumulative dose of antagonist. At the doses used, thymoxamine had a greater hypotensive effect than phentolamine. The regression lines did not differ significantly in slope ($P > 0{\cdot}9$).

Discussion

When the noradrenaline-induced blood pressure rises were plotted against log dose of noradrenaline (log x) the responses in the presence of the antagonists (y′) were moved to the right of the responses to noradrenaline alone (y) (Fig. 1). The first dose of the antagonists decreased the slope of the log dose-response line; in an isolated organ experiment this would suggest a non-competitive mode of antagonism. Increase in antagonist dose seemed to maintain the new slope, but the three log dose-response lines in the presence of antagonist were close together making further conclusions about the nature of the antagonism, from analogy with isolated organ experiments, more difficult. Thus it appears

that although a conventional log x versus y or y′ plot is known to be useful for analysing drug antagonisms on isolated tissues it yields limited information in the intact animal, in which the situation is more complex. Not only are injected drugs subjected to the processes of absorption, distribution and metabolism, but the measurements of arterial blood pressure are the resultants of the cardiac output and peripheral resistance influenced by the vasomotor compensatory reflexes in which noradrenaline released from sympathetic nerves plays a part. In the experiments here reported the situation was further complicated by the fact that the baseline blood pressure was itself lowered (further to activate the compensatory reflexes) by each succeeding dose of α-blocking agent, by an amount which was proportional to the logarithm of the cumulative dose of antagonist (Fig. 4). It is a common experience that a change in baseline of a measured function alters the extent to which that function may be modified

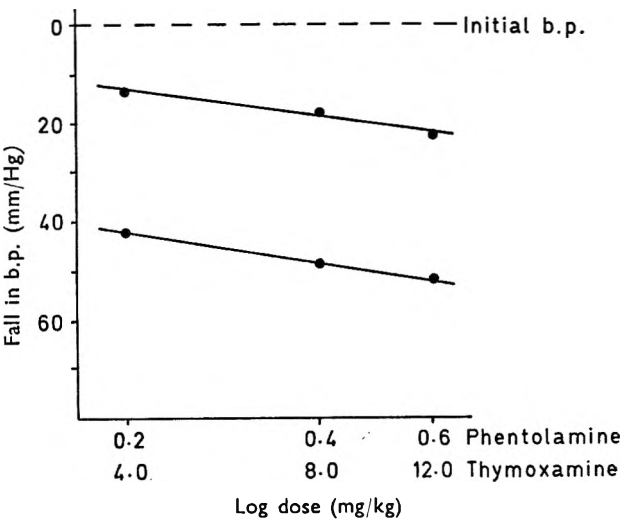

FIG. 4. The effect of thymoxamine or phentolamine on the blood pressure of the conscious cat. Ordinate: decrease in blood pressure in mm Hg (the resting blood pressure of the cat before antagonist minus the stable blood pressure after each succeeding dose of antagonist). Abscissa: the cumulative dose of the intravenously-administered α-blocking agent in mg/kg on a logarithmic scale. For phentolamine (upper line) each point is a mean of seven observations on five cats; for thymoxamine (lower line) each point is a mean of six observations on another five cats.

by drugs (Wilder, 1957a,b). The implication in this situation is that at a lower baseline blood pressure, the blood-pressure-raising effect of a dose of noradrenaline will be greater than the effect of the same dose of noradrenaline at a higher baseline blood pressure. Thus the closeness of the log dose-response lines in the presence of the antagonists could be, at least in part, a consequence of the effect of the antagonists on the baseline blood pressure.

Despite the complexity of the situation, Chen & Russell (1950) showed that by appropriate graphical treatment of the results it was possible to

analyse drug antagonism of the blood pressure responses to adrenaline in anaesthetized dogs. They used the graphical solutions of Lineweaver & Burk (1934) derived from the kinetics of enzyme-substrate and enzyme inhibitor combinations. For enzyme reactions, a plot of the reciprocal of the velocity of the reaction against the reciprocal of the substrate concentration yields a straight line. In the presence of competitive inhibitors the slope of the line is increased without change of intercept on the velocity co-ordinate, whereas non-competitive inhibitors increase the slope and raise the intercept. Chen & Russell (1950) showed in the anaesthetized dog that the slope of the lines obtained by plotting 1/dose against 1/response for adrenaline, increased in slope with cumulative increase in dose of yohimbine or with low doses of SY-28*, but the lines all had a common intercept with the line for adrenaline alone (by analogy with *in vitro* enzyme experiments this might indicate competitive antagonism) whereas the line for a large dose of SY-28 had a raised intercept on the 1/response co-ordinate (analogous with non-competitive antagonism in the enzyme experiments of Lineweaver & Burk, 1934).

When the present results were graphed as Lineweaver and Burk plots, 1/x versus 1/y or 1/y′ (Fig. 2), it was apparent that the first dose of thymoxamine or phentolamine increased the slope of the line and raised the intercept on the 1/response co-ordinate, indicating non-competitive antagonism. Thereafter, cumulative increases in antagonist dose gave lines showing progressively increased slopes but the intercepts were common with that of the line for the first dose of antagonist, indicating that further antagonism could be competitive. One exception to this general finding was seen when the total dose of phentolamine had reached 0·6 mg/kg. The response to the lowest dose of noradrenaline (0·1 μg/kg) was not linear with the responses to the other three doses. Such a breakdown of the linear relation between 1/x and 1/y′ was also found by Chen & Russell (1950) and by Matsumoto & Kumoi (1958) for acetylcholine-atropine combinations.

Matsumoto & Kumoi (1958) made a further analysis of the situation in which there was a change of intercept on the y axis and claimed that non-competitive antagonism could be differentiated from uncompetitive antagonism whereas such differentiation was not possible with log x versus y and y′ and 1/x versus 1/y and 1/y′ plots. By using the more complex co-ordinates log x and log $[y/(y_{max} - y)]$ or log $[y'/(y'_{max} - y')]$, derived from the considerations of Clark (1937) & Gaddum (1943),they showed that the position of the line in the presence of antagonist relative to that for the agonist alone allowed a further separation of the types of antagonism. When the present results for noradrenaline alone and for noradrenaline in the presence of the first dose of thymoxamine or phentolamine were treated in this way, the line for noradrenaline in the presence of the antagonist was moved to a position above and parallel to the line for noradrenaline alone (Fig. 3) conforming to the situation which Matsumoto & Kumoi (1958) held to be a combination of non-competitive and uncompetitive antagonism.

* *N*-(2-Bromoethyl)-*N*-ethylnaphth-1-ylmethylamine.

The broad similarities in the results of the quantitative analysis suggest that the mechanism of the noradrenaline antagonism exhibited by thymoxamine and phentolamine is the same. Thus it seems that a first dose of thymoxamine or phentolamine has a complex effect on the dynamics of a noradrenaline-induced blood pressure rise. This may in part be a reflection of the fact that for either antagonist the first dose had the greatest single effect in lowering the blood pressure (presumably by blockade of the effects of noradrenaline released from vasoconstrictor nerves) thereafter the dose increments effected smaller decreases in basal blood pressure. The differences between anaesthetized and unanaesthetized cats in their responses to catecholamines have been described by Thuránszky (1966a) and may be sufficient in themselves to account for the differences between our results with α-blocking agents and the results reported by Chen & Russell (1950) and those by Matsumoto & Kumoi (1958). It would appear from our experiments that after antagonism with an adrenergic blocking agent has been initiated in the conscious cat, or when the animal is anaesthetized, the antagonism has the characteristics associated with competitive blockade. These results do not provide direct evidence for the precise site of agonist-antagonist interaction. Although it is likely that most of the antagonism measured in these experiments was occurring at vascular α-receptors, the complexity of the factors controlling blood pressure in the conscious cat make it unlikely that α-blockade was the only mechanism involved. The antagonist may have been acting at sites other than the vascular α-receptors where the antagonism may be competitive or non-competitive. The basic assumption that the observed response was proportional to the number of receptors occupied by noradrenaline may not be valid. It is also possible that the different vascular beds in the animal have different affinities for the antagonists. Finally, as Lineweaver & Burk (1934) emphasized, mathematical manipulation of experimental data only indicates what mechanisms *may* be but not necessarily *are* involved in the situation examined.

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Methaemoglobin formation induced by aromatic amines and amides

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Two series of anilides have been examined for their ability to induce the formation of methaemoglobin in cats. The first series is one in which the aniline was acylated by a series of acids and the second is one in which alkyl groups were substituted on the benzene ring and the acyl moiety kept as the acetyl group. In neither series was any correlation found between the methaemoglobin-forming ability and stability of the amide group. In the first series, as the size of the acyl group was increased so the activity rose to a peak and then declined. It is suggested that this phenomenon is related to absorption of the compounds from the gastrointestinal tract. In the second series a parallelism in response was observed between amides given orally and the corresponding amines administered intravenously, suggesting that the nature of the amine liberated on hydrolysis is the major determining factor in the methaemoglobin forming ability of amides.

AROMATIC amines and their derivatives are known to produce methaemoglobinaemia (Bodansky, 1951). Drugs such as phenacetin, paracetamol, acetanilide, some sulphonamides (Harris, 1963) and prilocaine (Scott, Owen & Richmond, 1964), which are all aromatic amides, have been reported to form methaemoglobin. Further oxidation of haemoglobin to irreversible degradation products, including sulphaemoglobin and Heinz bodies, has been observed with certain drugs (Allen & Jandl, 1961). Brodie & Axelrod (1948, 1949) showed that the formation of methaemoglobin by acetanilide and phenacetin in man is related to the concentration of free amine in the blood, but the free amines do not oxidize haemoglobin *in vitro* (Prankerd, 1961). Methaemoglobin is thought to be formed *in vivo* by an oxidation product of the amine (Brodie & Axelrod, 1949; Kiese, 1965). Thus the reactions involved in the formation of methaemoglobin by aromatic amides such as acetanilide are (i) hydrolysis of the amide to the corresponding amine, (ii) metabolism of the amine to the appropriate species, and (iii) oxidation of haemoglobin by the amine metabolite.

The concentration of the active metabolite in the red cell depends on the absorption, distribution and excretion characteristics, both of the amide and of its metabolites. Since erythrocytes possess reductase systems capable of reversing this oxidation reaction (Harris, 1963), the actual concentration of methaemoglobin within the red cell depends on the relative rates of its formation and reduction.

We have examined the effect of retardation of the hydrolysis of aromatic amides on methaemoglobin formation.

It has long been known that the rate of chemical hydrolysis of anilides is slowed by *ortho*-substituents (Semerano, 1931). Thomas & Stoker (1961) have shown that *ortho*-substitution of benzoic esters, which are hydrolysed by a similar mechanism to anilides, retards both hydroxylation

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and esterase catalysed hydrolysis. Lignocaine, a derivative of 2,6-dimethylaniline, has been shown to be extremely refractory to chemical hydrolysis (Bullock & Grundy, 1955). Davis (1909) showed that increasing the length and bulk of the acid group of an anilide slowed the rate of its hydrolysis.

The effects of substitution in both the acid and amine parts of the acetanilide molecule on methaemoglobin formation have been examined.

Experimental

CHEMICAL

All compounds, except paracetamol, phenacetin and lignocaine, were prepared by standard methods and they are listed, together with physical constants, in Table 1.

TABLE 1. ANALYSIS AND MELTING POINTS OF ANILIDES AND AMINE HYDROCHLORIDES

Compound	M.p. °C	Found			Required		
		C	H	N	C	H	N
Acetanilide	114	71·3	6·7	10·4	71·1	6·7	10·4
2-Methylacetanilide	110	72·5	7·4	9·5	72·5	7·4	9·4
3-Methylacetanilide	65–6	72·8	7·6	9·4	72·5	7·4	9·4
4-Methylacetanilide	153	72·6	7·2	9·3	72·5	7·4	9·4
2,6-Dimethylacetanilide	177	73·4	7·9	8·5	73·6	8·0	8·6
2,6-Dimethylpropionanilide	122	74·9	8·5	8·1	74·6	8·5	7·9
2,6-Dimethylbutyranilide	135–7	75·7	8·8	7·6	75·4	8·9	7·3
2,6-Dimethylvaleranilide	72–3	76·1	9·3	7·4	76·1	9·3	7·0
2,6-Dimethylbenzanilide	168	80·0	6·6	6·1	80·0	6·7	6·2
Propionanilide	105	72·3	7·4	9·4	72·5	7·4	9·4
Butyranilide	96	73·5	7·8	8·8	73·6	8·0	8·6
Isobutyranilide	105	74·0	8·0	9·0	73·6	8·0	8·6
Valeranilide	62	75·0	8·8	8·2	74·6	8·5	7·9
Hexanilide	93–5	75·2	8·8	7·6	75·4	8·9	7·3
Octanilide	53	76·4	9·8	6·6	76·7	9·6	6·4
Decanilide	66–7	77·7	10·2	6·0	77·7	10·1	5·7
Dodecanilide	76–7	78·5	10·4	5·1	78·5	10·5	5·1
Aniline hydrochloride	198	55·9	6·6	10·6	55·6	6·2	10·8
2-Methylaniline hydrochloride	215–6	58·7	7·0	10·0	58·5	7·0	9·8
3-Methylaniline hydrochloride	208	58·6	7·2	9·6	58·5	7·0	9·8
4-Methylaniline hydrochloride	243	58·2	7·0	9·8	58·5	7·0	9·8
2,6-Dimethylaniline hydrochloride	179	61·0	7·6	8·7	61·0	7·6	8·9

Determination of water solubility. An amount of the compound, much in excess of its expected water solubility, was mixed with fresh glass-distilled water and the mixture slowly rotated in a water bath at 25° ± 0·1° for 21 days. The mixture was then filtered and duplicate samples of the filtrate measured into tared flasks. Water was removed from the samples by freeze drying and the flasks were dried in an oven at 80° for 30 min, allowed to cool and weighed. The method was checked by applying it to solutions of known concentration when quantitative recovery of the amides was obtained. A plot of log solubility of the amides against the number of carbon atoms in the acyl group is a straight line. The values obtained are given in Table 2.

BIOLOGICAL

Selection of species. It has been shown that different species form various amounts of methaemoglobin after administration of aromatic

amines and amides. For both acetanilide and phenacetin the order has been found to be cat>man>dog>rat (Lester, 1943; Spicer, 1950). We used cats and dogs.

TABLE 2. MAXIMUM METHAEMOGLOBIN (%) FORMED IN CATS AFTER ORAL ADMINISTRATION OF A HOMOLOGOUS SERIES OF ANILIDES. DOSE 1 M-MOLE/KG. EACH VALUE IS THE MEAN OF THE VALUES OBTAINED FROM 6 CATS. HYDROLYSIS RATES GIVEN AS TIME IN HR FOR 50% HYDROLYSIS. SOLUBILITY IN WATER MOLE/LITRE AT 25°

Compound	Mean max. % MetHb formed	s.d.	Base catalysed hydrolysis rate*	Solubility in water mole/litre 25°
Acetanilide	71·8	57·7	3·8	0·0464
Propionanilide	84·2	164·0	6·3	0·0 32
Isobutyranilide	87·0	127·3	30·7	0·0090
n-Butyranilide	88·0	49·9	13·0	0·0057
Valeranilide	75·6	11·4	18·5	0·0021
Hexanilide	75·7	34·9		0·00055
Octanilide	69·3	72·3		
Decanilide	46·1	184·9		
Dodecanilide	18·5	489·5		

* Davis, 1909.

Determination of methaemoglobin. Methaemoglobin was estimated by a modification of the photometric cyanomethaemoglobin method of Evelyn & Malloy (1938). Solutions were prepared as described by Hawke, Oser & Summerson (1954). Light absorption measurements were made on a Hilger and Watts Uvispek equipped with a quartz prism using matched glass cuvettes of 1 cm optical path. The absolute amounts of haem pigments in the samples were not determined and the results are expressed as percentage haemoglobin converted to methaemoglobin. Where irreversible oxidation occurs the concentration of both haemoglobin and methaemoglobin may be lowered and the values obtained by this method are suspect. Examination for irreversible oxidation products of haemoglobin was made using the method of Harley, J. D. & Robin, H. (personal communication). For aniline, at a dose level of 0·25 m-mole/kg in cats, the extent of oxidative degradation was found to be insignificant.

Procedure. Food was withheld from the animals for 16 hr before the start of an experiment. The animals were unanaesthetized, the cats being restrained in jackets made from strong cloth.

Compounds, which were administered orally, were reduced to fine powder and suspended in a 1% methylcellulose mucilage. Solutions of the amine hydrochlorides, which were given intravenously, were adjusted to pH 5·5 and made isotonic with sodium chloride. Injections were made slowly into the femoral vein with a dose volume of approximately 10 ml.

Blood samples were taken from the femoral vein just before administration of a drug and at each hour afterwards for 5 hr with the amines and 6 hr with the amides.

Methaemoglobin estimations were made in duplicate on each sample. Dilution of 0·1 ml quantities of heparinized blood in 10 ml of M/50 phosphate buffer was made immediately after withdrawal of the samples.

The lysis of the cells which occurs on dilution prevents reduction of methaemoglobin by cellular enzymes (Onji & Tyuma, 1965).

At least 5 cats were used for each compound at each dose level. Two dogs were used for each compound and a crossover procedure was adopted.

Results and discussion

Twenty cats were given aniline intravenously at a single dose level (0·0625 m-mole/kg) and the amount of methaemoglobin present in the blood determined hourly for 5 hr, by which time the response had passed its peak. The maximum amounts of methaemoglobin formed follow an approximately normal distribution (Fig. 1). The experiment was repeated one week later on 5 of the cats. The maximum responses for each cat

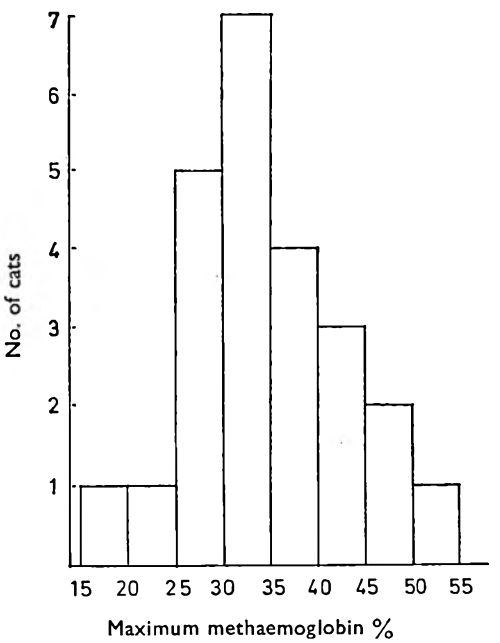

FIG. 1. Maximum methaemoglobin percentage formed in cats after intravenous administration of aniline solution at pH 5·5. Dose 0·0625 m-mole/kg.

on the two occasions were in good agreement. For both aniline and acetanilide a linear dependence of mean response upon log dose was shown to exist (Fig. 2). The slopes and positions of the regression lines were calculated by standard statistical methods (Burn, Finney & Goodwin, 1950). Similar relationships were shown to exist for the methylanilines and the methylanilides.

Intraperitoneal injection of acetanilide in a 30% ethanolic solution resulted in the formation of less methaemoglobin than when the same dose was given orally. Because of this the intraperitoneal route was not used subsequently.

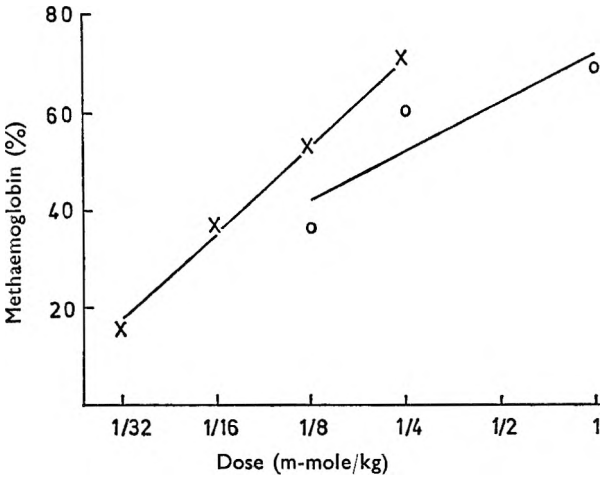

FIG. 2. The relationship between the log of the dose and the mean maximum percentage of methaemoglobin formed in cats after intravenous administration of aniline solution and oral administration of acetanilide suspension. Each point is the mean of the values obtained from 5 cats. Aniline ×——×, acetanilide O——O.

ANILIDES

The mean maximum methaemoglobin responses obtained for a series of anilides administered orally to cats at the same molar dose level are listed in Table 2. Six cats were used for each compound. The time course for methaemoglobin production by acetanilide, *n*-butyranilide and decanilide is shown in Fig. 3.

For the series from acetanilide to dodecanilide, analysis of variance showed that the response differs ($F_{8,45} = 23{\cdot}18$; $P < 0{\cdot}1$) with a peak response for the compounds propionanilide, *n*-butyranilide and isobutyranilide. Though the variances cannot be considered equal, the conclusion that the means differ with a peak response around butyranilide seems fairly sound. There is no apparent correlation between the stability of the anilides to chemical hydrolysis (Table 2) and the methaemoglobin response. Hydrolysis is therefore unlikely to be the rate-limiting step in methaemoglobin formation by these compounds. Since the same amine, aniline, is liberated on hydrolysis of anilides, differences in the methaemoglobin forming ability of the individual members of the series is presumably due to variation in the concentration of aniline available for further metabolism to the active species.

The compounds become less water soluble as the homologous series is ascended (Table 2) and so the oil-water partition coefficients would be expected to rise (Albert, 1960). It is also suggested by Albert that the lipid-water solubility characteristics of drugs influence their rates of dissolution, absorption and distribution within the tissues, both to sites of metabolism and sites of loss.

With this series it would appear that as the series is ascended a more favourable lipid-water solubility ratio is achieved with an optimum value

for methaemoglobin production around butyranilide. Thereafter, as water solubility decreases so does the methaemoglobin forming ability. At a limiting water solubility the dissolution rate of a compound will become significant in the regulation of absorption. In this series of experiments the particle size of the material was not controlled in any way and further work is being done on this aspect of the problem.

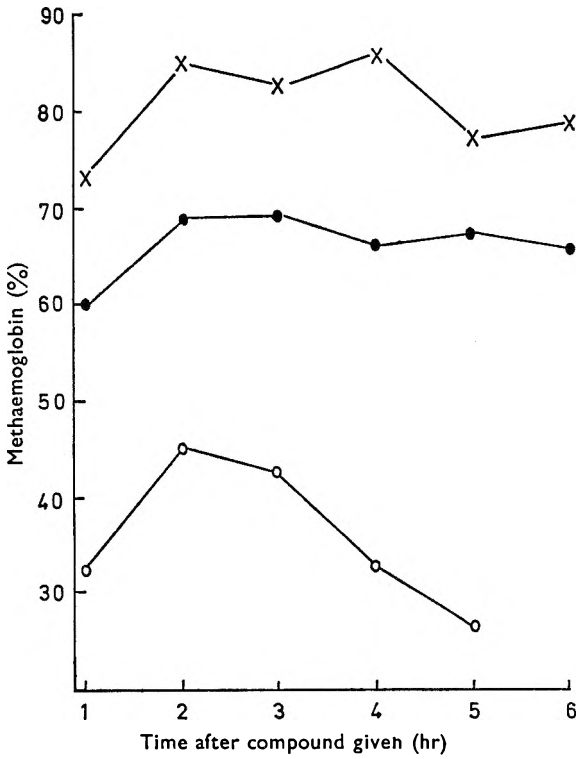

FIG. 3. The mean methaemoglobin percentage produced in cats at hourly intervals after administration of acetanilide ×——×, n-butyranilide ●——● and decanilide ○——○. Each point is the mean of the values obtained from 5 cats. Dose 1 m-mole/kg.

The individual variation in methaemoglobin response to dodecanilide was much greater than with the other anilides. This is possibly due to irregular absorption.

ANILINE AND MONOMETHYL SUBSTITUTED ANILINES

The mean maximum methaemoglobin responses obtained in cats for aniline and the methylanilines administered intravenously at the same dose level are given in Table 3. For treatment differences it was found $F = 9{\cdot}8$, indicating that the treatments differ. A Least Significant Difference test showed that it is due to 4-methylaniline forming much less methaemoglobin than the other amines.

TABLE 3. FORMATION OF METHAEMOGLOBIN AFTER INTRAVENOUS ADMINISTRATION OF ANILINE AND METHYLANILINES. DOSE 0·25 M-MOLE/KG

Compound	No. of cats used	Mean max. % MetHb formed	s.d.
Aniline	9	72·3	104·5
2-Methylaniline ..	9	70·1	136·1
3-Methylaniline ..	9	60·2	325·3
4-Methylaniline ..	8	39·6	187·1

It can be seen (Fig. 4) that the time course of the response to 3-methylaniline differs from that to aniline and 2-methylaniline. Although the maximum amounts of methaemoglobin formed do not differ significantly for the three compounds, the rate of methaemoglobin formation after 3-methylaniline is much slower. This difference in rate was also observed with the corresponding acetylated amines when they were administered orally.

The same order of response (Table 5) occurred when these compounds were administered to the dog.

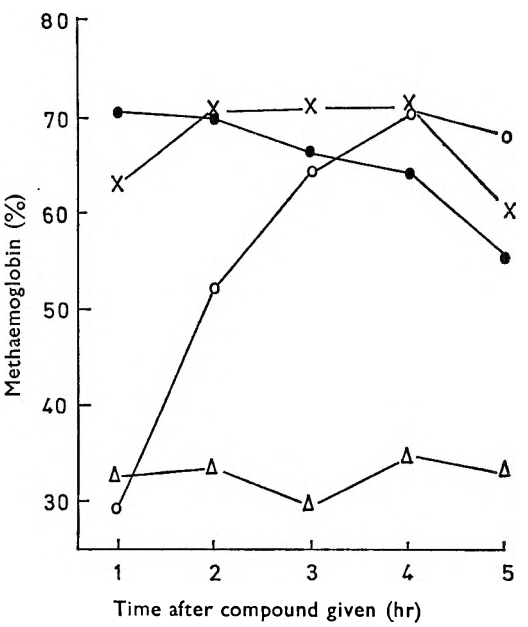

FIG. 4. The mean methaemoglobin percentage produced in cats at hourly intervals after intravenous administration of solutions of aniline ●——●, 2-methylaniline ×——×, 3-methylaniline ○——○, 4-methylaniline △——△. Each point is the mean of the values obtained from 5 cats. Dose 0·25 m-mole/kg.

SUBSTITUTED ANILIDES

The results obtained for this series in the cat are shown in Table 4. The variances for the treatments were not found to be homogeneous, therefore reservations must be made in interpreting the F value of 106.

It does seem large enough to admit the possibility of real differences in treatments. Further, when the treatment sum of squares was broken down for orthogonal components the major contribution was found to come from 4-methylacetanilide versus the rest. Thus it was concluded that 4-methylacetanilide forms less methaemoglobin than the rest. There was also an indication that 2- and 3-methylacetanilide form more methaemoglobin than acetanilide, phenacetin and paracetamol. Other comparisons showed that acetanilide, phenacetin and paracetamol formed the same amount of methaemoglobin.

TABLE 4. MAXIMUM METHAEMOGLOBIN (%) FORMED IN CATS AFTER ORAL ADMINISTRATION OF SOME ACETANILIDE DERIVATIVES. DOSE 1 M-MOLE/KG. EACH VALUE IS THE MEAN OF VALUES OBTAINED FROM 5 CATS. SOLUBILITY IN WATER MOLE/LITRE AT 25° AND RELATIVE RATES OF HYDROLYSIS ARE GIVEN

Compound	Mean max. % MetHb formed	s.d.	Solubility in water mole/litre 25°	Hydrolysis rate Acetanilide = 1*
Acetanilide	70·5	59·6	0·0464	1·00
2-Methylacetanilide	79·7	168·1	0·0695	0·42
3-Methylacetanilide	83·8	41·3	0·0403	0·89
4-Methylacetanilide	34·5	526·3	0·0069	0·98
Paracetamol	64·5	55·3		
Phenacetin	72·5	19·3		

* Semerano (1931).

Since the water solubility of the simple anilides was considered to be the most important factor influencing their methaemoglobin-forming ability, it is interesting to note that 4-methylacetanilide has a water solubility comparable with that of butyranilide, the most active of the anilides. In fact, neither the water solubility nor hydrolysis rate (Table 4) appears to be important with these compounds. The activities of the acetylated methylanilines correlate with those of the corresponding free amines in both the cat and the dog (Table 5).

TABLE 5. MAXIMUM METHAEMOGLOBIN (%) FORMED IN CAT AND DOG AFTER ADMINISTRATION OF SOME SUBSTITUTED ANILINE COMPOUNDS AND THE CORRESPONDING *N*-ACETYL DERIVATIVES

Compound	Mean max. % MetHb formed			
	Cat		Dog*	
	Amine 0·25 m-mole/kg i.v.	*N*-Acetyl 1 m-mole/kg oral	Amine 0·25 m-mole/kg i.v.	*N*-Acetyl 1 m-mole/kg oral
Aniline	72·3	70·5	30·3	34·1
2-Methylaniline	70·1	79·7	30·5	25·9
3-Methylaniline	60·2	83·8		
4-Methylaniline	39·6	34·5	2·6	11·1
2,6-Dimethylaniline	10·4	4·5	0	0

* Each value is the mean of two experiments in different dogs.

2,6-DIMETHYL SUBSTITUTED ANILIDES

Less than 5% methaemoglobin is formed in the cat after oral administration of 2,6-dimethylacetanilide and the corresponding amine produces the same order of response (Table 5). Neither 2,6-dimethylacetanilide nor 2,6-dimethylaniline produces a response in the dog.

2,6-Dimethylacetanilide is stable towards both acidic and basic catalysed hydrolysis due to the "*ortho* effect", although an enzyme has been isolated from hog liver which is capable of hydrolysing 2,6-dimethylaniline amides (Krisch, 1963).

The stability of the amide bond is probably not of primary importance here. It may be noted that increasing chain length in this series has no significant effect on methaemoglobin production (Table 6). The methaemoglobin response to lignocaine, which is a 2,6-dimethylaniline amide, is shown in the same table.

TABLE 6. MAXIMUM METHAEMOGLOBIN (%) FORMED IN CATS AFTER THE ADMINISTRATION OF A SERIES OF 2,6-DIMETHYLANILINE AMIDES. DOSE 1 M-MOLE/KG ORALLY. FIVE CATS WERE USED FOR EACH COMPOUND EXCEPT 2,6-DIMETHYLBENZANILIDE WHEN ONLY ONE CAT WAS USED.

Compound	Mean max. % MetHb formed
2,6-Dimethylacetanilide	4·5
2,6-Dimethylpropionanilide ..	9·2
2,6-Dimethylbutyranilide ..	5·4
2,6-Dimethylvaleranilide	8·6
2,6-Dimethylbenzanilide ..	7·1
Lignocaine	8·0*

* Dose 0·2 m-mole/kg intravenous.

Thus changes in structure which produce profound retardation of the hydrolysis of aromatic amides do not have a commensurate effect on the methaemoglobin formation which follows their administration. The nature of the amine appears to be of prime importance in determining the amount of methaemoglobin formed by these compounds.

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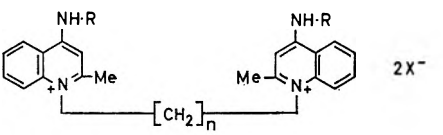
A note on a series of decamethylenebis[(4-substituted amino)quinaldinium] salts with potent antibacterial properties

W. A. COX AND L. H. C. LUNTS

A number of decamethylenebis[4-(substituted amino)quinaldinium acetates] have been prepared and certain of these showed markedly increased antibacterial activity compared with the unsubstituted compound. Local therapeutic activity, assessed by mouse protection tests, reached a maximum in the C_3–C_6 range of the *n*-alkylamino-homologues, but increased activity was associated with increased toxicity.

THE antibacterial properties of certain bis-quaternary heterocyclic compounds including the polymethylenebis(4-aminoquinaldinium) salts have been described in a number of publications reviewed by D'Arcy & Taylor (1961).



I

A number of derivatives of decamethylenebis(4-aminoquinaldinium) salts (I; n=10, R=H: dequalinium B.P.) have been prepared, together with a series of 4-alkylamino- and 4-cycloalkylamino-compounds. The synthesis and antimicrobial properties of these compounds are now described.

Materials and methods

CHEMISTRY

4-Alkylaminoquinaldines were prepared by reacting together 4-chloroquinaldine or 4-phenoxyquinaldine with an alkylamine in refluxing phenol. Details of hitherto unreported alkyl- and cycloalkyl-aminoquinaldines are given in Table 1.

The decamethylene bis-quaternary di-iodides (I; $n=10$, X=I) were made by refluxing 2 moles of base with decamethylene di-iodide in ethyl methyl ketone or in methyl isobutyl carbinol. The di-iodides were converted into water-soluble diacetates by reaction with silver acetate in methanol. These analysed generally as hydrated salts.

The bis-quaternary acetamido salt (compound 4) was prepared directly by acetylating dequalinium [decamethylenebis(4-aminoquinaldinium acetate): compound 1]. The salt was isolated as a di-iodide, the diacetate being obtained from it in the usual way.

Examples of these reactions are given in the Experimental section, and details of the salts are given in Table 2.

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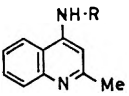
TABLE 1. 4-ALKYL- AND 4-CYCLOALKYL-AMINOQUINALDINES[1]



R	M.p. °C	Crystallization[2] solvent	Analyses					
			Found %			Required %		
			C	H	N	C	H	N
CH_2CH_3	180–181·5	E–W	77·6	7·7	14·65	77·4	7·6	15·0
$CH(CH_3)_2$	161–162·5	B	78·1	8·2	13·7	78·0	8·05	14·0
$CH_2{\cdot}CH{:}CH_2$[3] ..	145·5–146·5	B–P	78·9	7·0	13·85	78·7	7·1	14·1
$[CH_2]_3{\cdot}CH_3$	94–96	B–P	78·4	8·7	13·0	78·5	8·5	13·1
$[CH_2]_4{\cdot}CH_3$	106–107	B–P (b.p. 100–120°)	78·7	8·7	12·4	78·9	8·8	12·3
	130–132	B–P (b.p. 100–120°)	79·95	7·9	11·95	79·6	8·0	12·4
$CH(CH_3){\cdot}CH_2{\cdot}CH(CH_3)_2$	122–124	E–W	78·9	9·2	12·0	79·3	9·15	11·6
	150	B–P	79·9	8·1	11·2	79·95	8·4	11·7
$[CH_2]_6{\cdot}CH_3$	118–119	P (b.p. 100–120°)	79·6	8·8	11·05	79·6	9·4	10·9
$[CH_2]_7{\cdot}CH_3$	79·5–80·5	P (b.p. 60–80°)	79·9	9·7	10·0	79·95	9·7	10·4
$[CH_2]_2{\cdot}CH(CH_3){\cdot}CH_2{\cdot}C(CH_3)_3$[4]	118–120	B–P	70·7	9·55	9·1	71·2	10·1	8·75

[1] Prepared from 4-chloroquinaldine unless otherwise stated.
[2] E = Ethanol, W = Water, B = Benzene, P = Light petroleum, b.p. 40–60° unless stated otherwise.
[3] Prepared from 4-phenoxyquinaldine.
[4] Dihydrate.

BACTERIOSTATIC AND BACTERICIDAL EVALUATION

The *in vitro* bacteriostatic activity of the compounds was determined using a conventional tube dilution technique (Caldwell, Cox, D'Arcy & Rowe, 1961) against *Staphylococcus aureus* CN 491, *Escherichia coli* AH, *Proteus vulgaris* LH 14 and *Pseudomonas aeruginosa* NCTC 8203.

Bactericidal activity was assessed by a quantitative pour plate technique fulfilling the requirements of the British Standard method (1960). Samples were inactivated by dilution in suramin sodium 0·2% w/v (Antrypol, I.C.I. Ltd.).

CHEMOTHERAPEUTIC ACTIVITY

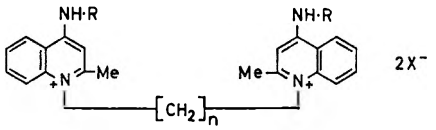
Protective tests were made in mice infected with *Staphylococcus aureus* 663. Cultures of this strain were grown overnight in Todd-Hewitt broth (37°). Culture suspensions in 2·5% hog mucin were injected intraperitoneally into groups of 10 mice (about 10^8 cells/mouse; minimal lethal dose, MLD × 10). Suspensions of the drugs in 10% gum acacia were given by the same route within 30 min of administering the culture. The numbers of mice surviving over 24 hr and 7 days were recorded.

Results

BACTERIOSTATIC AND BACTERICIDAL PROPERTIES

The effects of substitution in the 4-amino-group of dequalinium (compound 1) are summarized in Table 3. Introduction of an acetamido-group has little effect on bacteriostatic activity compared with the parent

TABLE 2. DECAMETHYLENEBIS[4-(SUBSTITUTED AMINO)QUINALDINIUM] SALTS



I

(n = 10; X = I or $CH_3 \cdot CO_2 \cdot 2H_2O$)

Comp. No.	R	Salt	Cryst. solvent[1]	M.p. °C	Analyses							
					Found %				Required %			
					C	H	I	N	C	H	I	N
	CH_3	Iodide	M–E	262–4	51·7	6·2	33·9	7·6	52·0	6·0	34·4	7·6
2		Acetate[2]	E–A	180–2	67·3	8·4	—	9·2	67·6	8·5	—	8·8
	$CH_2 \cdot CH_3$	Iodide	M–E	246	53·6	6·5	33·3	7·05	53·3	6·3	33·1	7·3
3		Acetate	E–A	159–160	69·1	8·4	—	8·3	68·45	8·7	—	8·4
	$CO \cdot CH_3$	Iodide	E	224–5	51·4	5·4	31·55	6·9	51·4	5·6	31·95	7·05
4		Acetate[3]	E	>360	69·0	7·85	—	8·3	69·3	7·65	—	8·3
	$[CH_2]_2 \cdot CH_3$	Iodide[4]	E	142–4	53·0	6·8	31·95	6·8	53·2	6·7	31·2	6·9
5		Acetate	Er–EA	111–2	68·3	9·1	—	7·7	69·1	9·0	—	8·1
	$CH_2 \cdot CH : CH_2$	Iodide[4]	M–E	134	53·25	6·4	31·2	7·1	53·5	6·2	31·4	6·9
6		Acetate	M–A	130–2	69·3	8·55	—	8·0	69·5	8·5	—	8·1
	$[CH_2]_3 \cdot CH_3$	Iodide	E	210–1	55·6	6·85	30·85	6·85	55·5	6·85	30·85	6·8
7		Acetate[5]	A–EA–Er	117–8			—	7·8			—	7·75
	$[CH_2]_4 \cdot CH_3$	Iodide	E	224–5	56·3	7·1	29·9	6·5	56·6	6·9	29·9	6·6
8		Acetate[6]	A	88–90	68·2	9·65	—	7·6	68·7	9·4	—	7·3
		Iodide	M–E	252–3	56·2	6·5	30·4	6·9	56·7	6·7	30·0	6·6
9		Acetate	A–Er	80–81	67·6	8·8	—	7·2	67·5	9·0	—	7·2
	$[CH_2]_5 \cdot CH_3$	Iodide	E	175–7	57·45	7·5	28·8	6·3	57·4	7·3	28·9	6·4
10		Acetate	A–Er	80·5–81	70·7	9·6	—	7·25	71·1	9·6	—	7·2
		Iodide	M	300–1	57·1	6·7	—	6·4	57·7	6·9	29·0	6·4
11		Acetate	A	122–4	71·25	9·4	—	7·8	71·3	9·1	—	7·2
	$[CH_2]_6 \cdot CH_3$	Iodide	M–E	198–200	58·05	7·7	28·05	6·7	58·3	7·6	28·0	6·2
12		Acetate	M–A–Er	112–4	71·6	10·6	—	7·15	71·5	9·7	—	6·9
	$CH(CH_3) \cdot CH_2 \cdot CH\ CH_3)_2$	Iodide	E–Er	231–2	57·5	7·5	28·1	6·2	57·4	7·3	28·9	6·4
13		Acetate	A–Er	65–7	71·0	10·5	—	7·2	71·1	9·6	—	7·2
	$[CH_2]_7 \cdot CH_3$	Iodide	M–E	168–171	58·9	7·6	27·35	6·1	59·1	7·8	27·1	6·0
14		Acetate	A	92–3	71·7	9·7	—	7·0	71·9	9·9	—	6·7

[1] M = Methanol, E = Ethanol, A = Acetone, Er = Ether, EA = Ethyl acetate.
[2] All acetates are dihydrates unless otherwise stated.
[3] Anhydrous.
[4] Monohydrate.
[5] Trihydrate.
[6] Tetrahydrate.

compound, whilst substitution by an allyl, cyclopentyl or cyclohexyl group results in slight increase in activity against *Staphylococcus aureus* and some improved activity against *Escherichia coli* and *Proteus vulgaris*.

The most significant changes in activity are associated with substitution by a straight chain alkyl group. From Fig. 1 it is seen that an increase in the number of substituent carbon atoms up to C_4 (n-butyl) results in improved antistaphylococcal activity whilst optimal activity against *Escherichia coli* is reached at C_5 (n-pentyl) and against *Proteus vulgaris* peak activity is shown at C_7 (n-heptyl).

The series of decamethylenebis[4-(substituted amino)quinaldinium] compounds were compared for their bactericidal action against *Staphylococcus aureus* and *Escherichia coli* and from the results, summarized in Table 3, it is generally apparent that increased activity is associated with increase in the size of the substituent group.

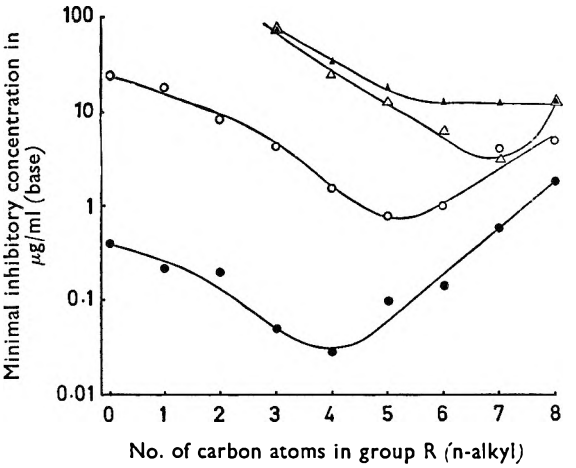


FIG. 1. Bacteriostatic activity of decamethylenebis[(4-n-alkylamino)quinaldinium acetates] against *Staphylococcus aureus* CN491 (●—●), *Escherichia coli* AH (○—○), *Proteus vulgaris* LH14 (△—△) and *Pseudomonas aeruginosa* NCTC8203 (▲—▲) after 24 hr incubation at 37°.

LOCAL CHEMOTHERAPEUTIC ACTIVITY

The activity of the decamethylenebis(4-alkylaminoquinaldinium) salts has been compared with dequalinium (compound 1) in intraperitoneal protection tests in mice against *Staphylococcus aureus*. The results, summarized in Table 4, showed that maximum therapeutic activity occurred when the n-alkyl substituent contained three to six carbon atoms (compounds, 5, 8, 10). An associated increase in toxicity is found with increased chain length. The ratio of the curative dose to the equivalent

TABLE 3. BACTERIOSTATIC AND BACTERICIDAL ACTION OF DECAMETHYLENEBIS[4-(SUBSTITUTED AMINO)QUINALDINIUM ACETATES]

Comp. No.	Minimal Inhibitory Concentration μg/ml (base) at 24 hr				Bactericidal activity* Mean % reduction on viable count	
	Staph. aureus CN491	*E. coli* AH	*Ps. aeruginosa* 8203	*Pr. vulgaris* LH14	*Staph. aureus* CN491 10^{-6}M	*E. coli* AH 10^{-5}M
1 (Dequalinium)	0·3	25	>100	100	15·87	99·87
2	0·22	17·7	>100	>100	21·43	94·98
3	0·19	8·8	>100	>100	53·47	91·89
4	0·17	50	>100	>100	59·01	99·92
5	0·05	4·4	75	75	0	98·36
6	0·04	12·5	100	100	7·14	69·8
7	0·03	1·5	35	25	55·44	99·79
8	0·1	0·8	17·7	12·5	91·88	99·96
9	0·4	1·1	>100	35	45·87	99·09
10	0·16	1·0	12·5	6·2	97·52	99·99
11	0·17	1·5	>100	25	0	99·96
12	0·6	4·4	12·5	3·1	97·10	99·99
13	0·15	0·6	35	4·4	—	—
14	1·8	4·4	12·5	12·5	96·34	99·99

* After 15 min contact at 20° C.

toxic dose indicates that the optimum response, in the series of 4 n-alkylamino-compounds occurred when the n-alkyl substituent contained six carbon atoms.

TABLE 4. PROTECTION OF MICE BY DECAMETHYLENEBIS[4-(SUBSTITUTED AMINO)-QUINALDINIUM ACETATES] AND POLYMETHYLENEBIS(4-AMINOQUINALDINIUM ACETATES). Compounds administered intraperitoneally 30 min after intraperitoneal inoculation of culture

Compound No.	Formula I	*Staphylococcus aureus* 663		
		ED75 (7 days) mg/kg	LD75* mg/kg	ED75/LD75 ratio
1 (Dequalinium)	R = H; n = 10	4·0	9·3	0·43
2	R = CH_3; n = 10	2·4	5·1	0·46
5	R = C_3H_7; n = 10	1·7	5·9	0·29
8	R = C_6H_{11}; n = 10	1·0	4·2	0·24
10	R = C_8H_{18}; n = 10	2·2	4·6	0·48
14	R = C_8H_{17}; n = 10	>10 (Toxic)	<0·6	—
—	R = H n = 14	>10 (Toxic)	—	—
—	R = H n = 16	>10 ,,	—	—
—	R = H n = 18	>10 ,,	—	—

* LD75 assessed in infected animals.

Discussion

The group of decamethylenebis[4-(substituted amino)quinaldinium] salts show significant changes in the peak of bacteriostatic activity against different bacterial species which are associated with an increase in the size of the alkyl substituent. There is little difference in activity between straight-chain and branched-chain alkyl-substituted compounds (cf. compounds 10 and 13) when the substituents are of approximately similar size. The obvious effects of allyl substitution (compound 6) are not apparent in bacteriostatic results but considerable reduction in bactericidal activity is shown and similar effects are seen with the cycloalkyl compounds (9, 11). These cycloalkyl compounds are significantly less active than the corresponding n-alkyl compounds (8, 10).

The activity of the dequalinium salts in protecting mice locally against intraperitoneal infection with *Staphylococcus aureus* was described by Babbs, Collier, Austin, Potter & Taylor (1956). The ability of the decamethylenebis(4-n-alkylaminoquinaldinium) salts to protect mice in similar experiments runs parallel with the bacteriostatic activity of these compounds.

Experimental

4-Heptylaminoquinaldine. A mixture of 4-chloroquinaldine (10·0 g) heptylamine (5·5 g) and phenol (10·0 g) was heated in an oil bath at 180°. After an initial exothermic reaction, which occurred when the mixture was at about 140°, the heating was continued for 4 hr. When cool, the mixture was poured into sodium hydroxide solution (20%: 200 ml) to precipitate an orange gum, which solidified. This was separated by filtration, washed, dried and crystallized from light petroleum (b.p. 100–120°) to give the base as a pink solid (7·5 g), m.p. 115–120°. After two recrystallizations, pale pink rods, m.p. 118°–119°, were obtained.

Decamethylenebis(4-heptylaminoquinaldinium iodide). 4-Heptylaminoquinaldine (2·85 g) and decamethylene di-iodide (2·0 g) in ethyl methyl ketone (20 ml) were refluxed for 96 hr. The mixture was cooled, filtered and the residue was washed successively with ethyl methyl ketone, acetone, warm water, acetone and ether to leave the *quaternary salt* as a pale purple solid (2·68 g), m.p. 190–192°. After three recrystallizations, with decolorization by charcoal, from a mixture of methanol and ethanol, buff coloured plates, m.p. 198–200°, were formed.

Decamethylenebis(4-heptylaminoquinaldinium acetate). The above di-iodide (3·63 g), dissolved in warm methanol (100 ml), was stirred with silver acetate (1·34 g) for 2 hr. The precipitated silver iodide (1·90 g) was removed and, after being clarified by filtration through diatomaceous earth, the solution was evaporated under reduced pressure. On addition of dry ether the residual oil slowly gave a pale pink solid (2·79 g), m.p. 107–108° (decomp.). This was purified by being dissolved in a mixture of acetone (100 ml) and methanol (10 ml) and treatment with charcoal. The solution was concentrated to 50 ml, dry ether (50 ml) was added and the oil which was obtained was triturated with dry ether to give the *bis-quaternary diacetate* as a pink solid (2·06 g), m.p. 111–112° (decomp.), raised to m.p. 112–114° (decomp.) on subsequent similar treatment.

Decamethylenebis(4-acetamidoquinaldinium iodide). Decamethylenebis-(4-aminoquinaldinium acetate) (10 g), acetic acid (50 ml) and acetic anhydride (50 ml) were heated under reflux for 26 hr. The solution was concentrated to a small volume and was treated with a solution of sodium iodide (10 g) in water. A dark oil, which slowly solidified, precipitated. It was separated from the aqueous solution and was extracted with hot ethanol (350 ml). The hot extract was boiled with charcoal, filtered, concentrated and allowed to cool. The *bis-quaternary di-iodide* separated as a yellow solid (5·3 g), m.p. 224° (decomp.), which was twice recrystallized from ethanol to give dark yellow plates, m.p. 224–225° (decomp.).

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A method for the quantitative determination of the total concentration of radiopaque agents in plasma

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The total concentration of radiopaque agents in plasma can be determined spectrophotometrically after elimination of interfering factors using a double column filled with cross-linked dextran-gel Sephadex G-25c and Se-Sephadex 25c. The method gives a linear relationship between the total concentration in the sample and the maximum concentration in the eluate and is sensitive to about 50 μg/ml of radiopaque agent.

IN investigations of the excretion pattern of radiopaque agents it is necessary to know the fraction of the total plasma concentration of the agent bound to plasma protein. This may be obtained using dialysis or ultrafiltration, from the free concentration in equilibrium and the quantity of agent originally applied to the system (Goldbaum & Smith, 1954; McMenamy & Oncley, 1958; Davison & Smith, 1961) but this is possible only when the drug or agent is not adsorbed on a membrane. We found radiopaque agents to be adsorbed (8–18 mg from concentrations of 1·2 mg/ml) onto Visking tubing (1·4 cm × 8 cm) and collodion (5 cm diam. × 6 cm) and the adsorption to depend on the concentration of the agent and to vary with the individual membrane used. Thus to determine the plasma protein binding of the radiopaque substance (Langecker, Harwart & Junkmann, 1953; Lasser, Farr, Fujimagari & Tripp, 1962), we had to measure the total concentration in the plasma or protein phase and the free concentration in the protein-free dialysate, at equilibrium. Therefore for *in vivo* experiments, and also for the determination of protein binding in *in vitro* experiments, we needed a method to measure the total concentration of radiopaque agent in the plasma or protein phase.

The agents can be determined spectrophotometrically but it is first necessary to separate them from plasma proteins and protein fragments, especially creatinine, which interfere with spectrophotometric determinations. It seemed probable that as the dextran gel Sephadex in a column had been shown to separate molecules of different sizes (Porath & Flodin, 1959; Porath, 1960; Barlow, Firemark & Roth, 1962), this approach promised a solution to the problem of eliminating interfering material. If, and how far, a dissociation of the protein-bound radiopaque agents can be obtained by this method, must be investigated (Barlow & others, 1962; Jacobsson & Widström, 1962; Lissitzky, Bismuth & Rolland, 1962).

For a column filled with Sephadex acting as a sieve for molecules of different size

$$K_D = \frac{V_e - V_o}{V_i}$$ (Barlow & others, 1962)

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where the parameters of the column are:

V_0 = the water outside the gel grains, V_i = the water within the gel grains, V_e = the volume of eluate required to deliver the peak concentration of the experimental substance, K_D = the distribution coefficient; this indicates the fraction of the inner volume accessible to a particular molecular species.

For low molecular weight compounds eluted on a neutral Sephadex column, $V_e = V_i + V_0$, so $K_D = 1$; deviations from the $K_D = 1$ value are due to adsorption onto the gel comparable with the adsorption which takes place by column chromatography on a column filled with cellulose. The gel is also available with ion-exchange properties and here, for low molecular weight compounds, deviations from the $K_D = 1$ value depend on the pK_a value of the compound and the pH of the eluent. Thus the ion-exchange gel not only differentiates on the basis of the dimensions of the molecules but also on basic or acidic properties (Porath & Lindner, 1961; Carnegie, 1961; Scheffer, Kikuth & Lorenz, 1965).

If a mixture from which acids or bases have to be separated contains proteins, the choice of the pH of the eluent is limited because the proteins may precipitate. For this reason these must first be removed in neutral conditions, therefore a neutral Sephadex column is required. If the mixture then contains only one acidic compound to be measured, separation can be effected by a cation-exchanger, and for this purpose a Sephadex cation exchanger can be used.

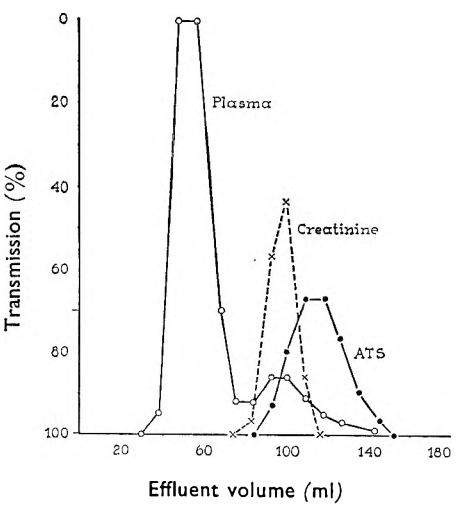

FIG. 1. The % transmission at 237 mμ as a function of the effluent volume. The curves represent the transmission pattern of 8 ml fractions of the eluate, obtained with 1 ml plasma, 1 ml creatinine (0·1 mg/ml in distilled water) and 1 ml acetrizoate sodium (ATS) (0·1 mg/ml in distilled water) respectively, eluted on a 40cm Sephadex G-25c column with Tyrode pH 7·6.

Note: The mean zone of plasma proteins does not overlap the zone of small molecules; for the separation of the small molecules a second step is necessary.

ELUTION ON NEUTRAL SEPHADEX (G-25C)

In experiments in which the elution of plasma proteins, creatinine and various radiopaque agents was examined singly (Fig. 1), we found (*a*) no permanent adsorption of the radiopaque agents onto the Sephadex (of the quantity of agent applied to the column 99·5–99·9% was recovered in the eluate), (*b*) the zone containing the plasma proteins did not overlap that containing the radiopaque agents, (*c*) the zone containing the agents and that containing creatinine as well as the zone obtained from the smaller molecules in plasma clearly overlapped. Therefore to determine radiopaque agents in plasma a second separation step was required. In experiments with combinations of plasma and radiopaque agents we found that there was no mutual influence as far as the elution zones are concerned; the minima in the light transmission in these zones remain unchanged. So a total dissociation of the radiopaque-protein complex is obtained. From the latter experiments the values for V_o, V_i and V_e, and consequently K_D can be calculated (see Table 1).

TABLE 1. EXPERIMENTAL DATA FOR THE RADIOPAQUE AGENTS ELUTED ON A SEPHADEX G-25C COLUMN

Sample placed	Start of the peak	Minimum of the peak		End of the protein peak		Vo	Vi*	Ve	KD
	EV in ml	EV in ml	% Trans-mission	EV in ml	% Trans-mission				
Blank plasma		40	1	60	96	40			
Creatinine, 0·4 mg/ml in H_2O		90	55				50	90	1·0*
Acetrizoate-Na, 0·4 mg/ml in H_2O	70	100	75					100	1·2
Acetrizoate-Na, 0·4 mg/ml in plasma ..	72	102	74	60	96			102	1·24
Iodohippurate-Na, 0·4 mg/ml in H_2O ..	82	112	84					112	1·44
Iodohippurate-Na, 0·4 mg/ml in plasma ..	83	110	84	65	97			110	1·40
Iodopyracet-Na, 0·4 mg/ml in H_2O ..	102	135	87					135	1·9
Iodopyracet-Na, 0·4 mg/ml in plasma ..	106	138	87	65	97			138	1·96
Diatrizoate-Na, 0·4 mg/ml in H_2O ..	65	88	68					88	0·96
Diatrizoate-Na, 0·4 mg/ml in plasma ..	64	90	66	60	96			90	1·0

EV = Effluent volume; 0·2 ml sample was placed in each case. Each determination was done in quadruplicate.

* The Vi values are calculated assuming that the KD value of creatinine is 1·0. As far as the radiopaque solutions in plasma are concerned the data of the peak minimum have reference to the radiopaque agent.

COMBINATION WITH A SE-SEPHADEX 25C COLUMN

As expected at lower pH values, there was a stronger retardation of the passage of basic and amphoteric compounds, while for the acids the tendency to pass unhindered was increased (Table 2). Therefore the column of neutral Sephadex was used with a column of the cation-exchange gel, Se-Sephadex 25c.

Materials and methods

Neutral Sephadex, Sephadex G-25c (approximate exclusion limit 5000 M.W.) and the cation exchanging Sephadex, Se-Sephadex-25c

(Pharmacia, Uppsala, Sweden) were used. Columns of 2 cm in diameter and 40 cm long for the neutral and 25 cm long for the cation exchanger Sephadex were combined, each having a sintered glass filter at the end (Fig. 2). After carefully washing the Sephadex in distilled water, followed by three washings in the elution fluid to be used, the column was filled as described by Porath (1960). Precipitation of plasma proteins was avoided by using a 0·08 M phosphate buffer. At the fixed pH 7·6, Tyrode 0·08 M can also be used.

TABLE 2. EXPERIMENTAL DATA OF COMPOUNDS ELUTED ON A SE-SEPHADEX-25C COLUMN, 25 CM OF LENGTH

Sample placed	Eluted with 0·08 M phosphate buffer		
	pH 7·4 Ve in ml	pH 6·0 Ve in ml	pH 5·0 Ve in ml
0·6 ml Plasma	30	30	30
0·6 ml Acetrizoate-Na, 0·1 mg/ml in H_2O	44	44	40
0·6 mg Creatinine, 0·1 mg/ml in H_2O	69	94	180

Each determination was done in triplicate.

In the experimental set-up (Fig. 2) the combined column is pretreated with 60 ml phosphate buffer pH 6·4. A 0·6 ml sample is placed on the top of the neutral (N) column with an Agla-micrometer supplied with a ball joint KVII. KII is turned to the position in which the N column is eluted with phosphate buffer pH 5·0. KI is turned into the position

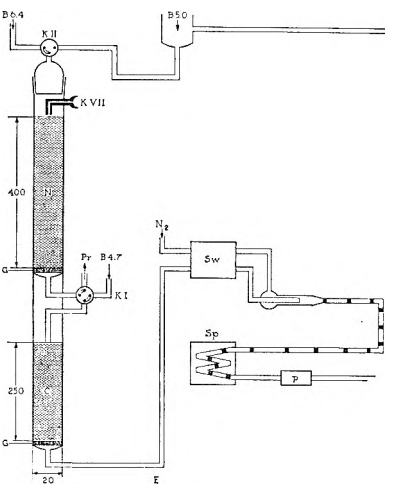

FIG. 2. Diagram of experimental set up: (N) Sephadex G-25c column; (C) Se-Sephadex 25c column; (G) glass filter; (KI) and (KII) multiway taps. (B 6·4) inlet phosphate buffer, pH 6·4, for pretreatment column (N), (B 5·0) inlet elution fluid, phosphate buffer pH 5·0, (KVII) socket joint for injection sample. The fixed height of the supply vessel, and the capillary tube of the inlet, makes it possible to place the sample on the column, while the column is eluting. (Pr) outlet protein fraction, (B 4·7) inlet phosphate buffer, pH 4·7, for pretreatment column (C); (SW) magnetic valve for inlet nitrogen from (N_2); (Sp) glass-spiral; (p) peristaltic pump used during the elution to give gradual filling (1·5 ml/min) of the glass-spiral with elution fluid. After filling, the spiral is connected to the flow cell of a spectrophotometer for recording.

shown in Fig. 2. While the protein fraction is removed in this way from the end of the N column (Pr in Fig. 2), the cation (C) column is pretreated with phosphate buffer pH 4·7. As soon as the desired effluent volume from the N column is removed—this volume depends on the K_D-value of the radiopaque agents (Table 1)—KI is turned to the position in which the N and C column are connected. Instead of taking separate samples, the per cent transmission in the elution fluid coming from the C column is measured continuously. The eluate is divided in small portions by nitrogen bubbles and stored in a glass spiral 2 mm diameter and 10 m long with a total content of 130 ml. When the fluid is passed through the column and stored in the spiral it can be passed at any suitable time and at any speed required through a flow cell placed in the Beckman DB spectrophotometer for the measurement of the per cent transmission. Before passing through the flow cell the nitrogen bubbles, which served to prevent convection and mixing of the fluid, are removed. The light transmission is recorded.

Results

The heights of the transmission peaks obtained with various samples of identical size and containing identical concentrations of the radiopaque agent were remarkably consistent. Table 3 gives an example of a comparison of the transmissions measured with 4 radiopaque agents dissolved in water and in plasma. Calibration curves for 4 radiopaque agents in water were prepared (see Fig. 3). With plasma samples identical curves

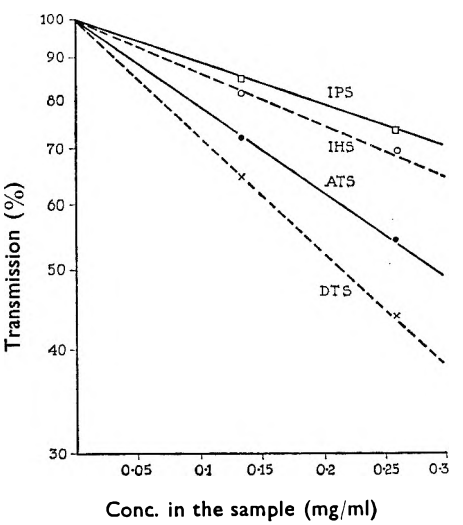

FIG. 3. The minimum in the transmission at 237 or 230 mμ measured for various radiopaque agents in the effluent of the double column system, expressed in percentage on a logarithmic scale as a function of the concentration of the radiopaque agent in the sample. (IPS) iodopyracet-sodium; (IHS) iodohippurate-sodium; (ATS) acetrizoate-sodium; (DTS) diatrizoate-sodium. Note the linearity in the relationship which indicates a practically 100% recovery of the drug and makes it possible to use the curves for calibration.

were obtained (see Table 3). By means of these calibration curves the concentration of the radiopaque agents in the unknown samples could be determined.

Calculation of a known concentration of 0·200 mg/ml with the aid of the calibration curves in Fig. 3, gives a value for the concentration of the sample of 0·199 ± 0·005. This result agrees with our other determinations and confirms that the determinations showed no deviation greater than 3% from the mean.

TABLE 3. CALIBRATION DATA FOR THE RADIOPAQUE AGENTS ON THE DOUBLE COLUMN

Sample placed on the column	Vol. removed from neutral column	% Transmission at the peak of the effluent fraction*			
		Sample conc. 0·1336 mg/ml H_2O	Sample conc. 0·1336 mg/ml plasma	Sample conc. 0·2506 mg/ml H_2O	Sample conc. 0·2506 mg/ml plasma
Acetrizoate-Na ..	70	72 (0·6)	72·4 (0·6)	55·2 (1·2)	
Creatinine	70				99 (0·3)
Iodopyracet-Na ..	100	84 (0·7)	83·3 (0·6)	75·5 (0·8)	
Creatinine	100				100 (0·1)
Iodohippurate-Na ..	80	83·2 (0·3)	82·3 (0·4)	71·4 (0·5)	
Creatinine	90				99 (0·2)
Diatrizoate-Na ..	60	64 (0·4)	64·5 (0·6)	45·8 (1·0)	
Creatinine	60				99 (0·3)

In all cases 0·6 ml sample was placed on the column. Each determination was done in quadruplicate.
* Mean value; the range is given in parentheses.

An important aspect of the data in Table 3 is that the concentrations of the radiopaque agents measured in the plasma samples were found to be identical to those in the water samples. This means that a total dissociation of the radiopaque agent from the proteins takes place and that with this method the total concentration, bound and unbound, of radiopaque agent is measured.

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Effects on the reproductive organs of feeding the non-ionic surfactant Triton X-100 to mice

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A series of feeding experiments has shown Triton X-100 to induce cystic degeneration in the mouse ovary. Parallel experiments in ovariectomized mice revealed no evidence that Triton X-100 possesses oestrogenic activity. Long term exposure of female mice to this surfactant did not impair fertility.

THERE is widespread interest in the possible biological effects of surfactants. Chapman & Short (1965) have reported a marked thickening of the interfollicular epidermis of sheep subjected to long term topical treatment with polysorbate. This is similar to the effects obtained in mice by Dammert (1957) and Setala, Merenmies, Stjernvall, Ahoy & Kajanne (1959). Our previous findings (Goldhammer, 1956; Goldhammer & McManus, 1960) with non-ionic surfactants in plants and rodents we thought could be the result of an alteration in cell-membrane permeability. To explore this possibility the alkyl aryl polyether alcohol surfactant Triton X-100 has been administered in the food or drinking water of mice to enable its effects on the reproductive organs to be assessed. The mouse ovary is known to respond to the topical application of Triton X-100.

Experiments and results

The criteria of assessment were the number of Graafian follicles at least 400 μ in diameter, the presence of haemorrhagic points and the number of corpora lutea. A concentration of up to 0·5% of Triton X-100 in food was well tolerated and subsequently 0·3% of the surfactant was used. No diarrhoea was noted at these levels. In drinking water a suitable concentration was 0·1%; concentrations up to 0·5% were tolerated but little consumed. Four experiments were conducted over a period of eight months.

Experiment I. Triton X100 was administered as a 0·1% solution (v/v) in drinking water to 47 female and 12 male mice over 8 months. The animals were 4–6 weeks old at the beginning of the experiment. Vaginal smears were taken from about two thirds of the females at least 3 times a week over the entire experimental period.

Experiment II. 49 female and 12 male mice were maintained on a diet of standard mouse cubes containing 0·3% (v/w) of Triton X-100 from the age of 4–5 weeks for 8 months. Vaginal smears were taken from all the females as above.

No gross alterations of the oestrous cycle and no microscopic changes in the testes of the treated animals were observed over the 8 month period.

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The ovaries showed enlarged "cysts", at least four to five times the size of the whole ovary. The incidence of this cystic condition was 23·4% and 40·8% for the "water" and "diet" experiments respectively compared with 20·8% in a control group of 98 animals ($P = 0{\cdot}15$ N.S.).

From each group ("diet" "water" and control) 18 females, chosen at random equal numbers being in oestrus, were killed at the end of the eight month period and ovarian sections made. These were cut 4μ thick at constant depth and were stained with haematoxylin-eosin. Follicles and corpora lutea at least 400 μ in diameter were counted in serial sections taking equal numbers of sections for each group. As tested by analysis of variance the differences were not statistically significant.

Examination of uterine sections from treated "diet" animals revealed pronounced cystic changes in this organ. In three treated "diet" mice and one treated "water" mouse, one ovary showed a haematoma-like gross enlargement not encountered in control animals.

Experiment III. 20 female mice were ovariectomized at 10 weeks. After three days, they were fed a diet of mouse cubes containing 0·3% of Triton X-100 (v/w) for 8 months to find if the surfactant possessed oestrogenic activity. Vaginal smears were taken either daily or four times a week. Four animals died in the first 4 months and of the remaining 16 two showed intermittent vaginal cornification over the last two months of the experiment. When the two animals were killed the uteri were swollen and hyperaemic and histological examination showed no remnant of ovarian tissue. The vaginal smears in the other 14 animals were always negative. These animals had atrophic uteri.

Experiment IV. This was to assess the effect of Triton X-100 upon fertility. Eighty 6-months old female mice were randomly allocated to 3 groups.

One group (30 mice) was fed on Triton X-100 (0·3% v/w) for 4 months and then transferred to control diet for the remaining 4 months. A second group (30 mice) received the same treatment in reverse order. The third group (20 mice) acted as a control. After 8 months the 3 groups were mated with 14 males which had been kept on a normal diet. During mating 4 females were caged with one male: the males were circulated among the groups of females. All females were fertile but cannibalism prevented comparison of litter size. In a subsequent experiment each female was housed individually and no difference was observed between the average litter size of the treated and the control animals.

Discussion

The phenomenon needing explanation is the increased incidence of cysts in the ovaries and uteri, occurring after prolonged feeding with Triton X-100.

It is assumed that some of the ingested surfactant reached the ovary. There is evidence that some surfactants of "Span" and "Tween" type are largely excreted unchanged (Treon, 1965) but we are unaware of any reports on the metabolism of the "Triton" type of surfactant.

Assuming that the effects seen are not due to impurities, we consider that any tenable explanation of them must include an action of Triton X-100 on living membranes. It is well known that cell membranes exercise selective permeability. Nissim (1964) and Hart & Nissim (1964) have suggested that the movement of glucose across intestinal cells is regulated by certain protein receptor sites, some situated on the membrane, some intracellular. They considered that the cationic surfactant cetrimide reacted with receptor proteins to alter the rate of glucose entry into the cells. It is possible that Triton X-100 may exert one or more of the following actions upon cell membranes: a reduction of surface tension at the membrane interface; an action upon receptor sites or pores; an alteration of lipid constitutents of the membrane structure (Booij, 1962). Various workers have postulated that hormones exert part or the whole of their effects, by influencing the activity of enzyme systems or by altering physical properties of limiting membranes (Smith & Williams, 1965). It could be that the Triton X-100 rendered ovarian cells more responsive to pituitary gonadotrophins. It is significant that the phenomenon took a long time to appear and that only some of the treated animals showed a response.

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The mechanism of the tranquillizing action of asarone from *Acorus calamus* Linn.

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Asarone, the active principle of the indigenous Indian plant *Acorus calamus* Linn., did not cause any change in the noradrenaline content of whole brain of rats. Unlike tetrabenazine, pretreatment with asarone failed to block the effect of reserpine on the spontaneous motor activity and ptosis of mice, as well as the conditioned avoidance response of trained rats. These findings show that, though in chemical structure asarone resembles a part of the reserpine molecule, its mechanism of action is different from this drug. In animals in which brain noradrenaline has been lowered by pretreatment with α-methyl-L-tyrosine, the effects of asarone, namely, hypothermia, potentiation of barbiturate hypnosis and specific blockade of conditioned avoidance response, were found to be markedly enhanced. It seems that the sedative effect of asarone is dependent on the depression of the ergotropic division of the hypothalamus.

ASARONE, the active principle from the Indian plant *Acorus calamus* Linn., has tranquillizing activity and is similar in some of its properties to reserpine and chlorpromazine (Dandiya & Sharma, 1962; Dandiya & Menon, 1963, 1964, 1965). However, an important difference between the actions of asarone and reserpine is the inability of asarone to deplete brain 5-hydroxytryptamine (Dandiya & Menon, 1963).

Since the chemical structure of asarone as elucidated by Baxter, Dandiya, Kandel, Okany & Walker (1960) (i.e. *cis* isomer of 2,4,5-trimethoxy-1-propenylbenzene) resembles a moiety of the reserpine molecule we have investigated whether reserpine and asarone produce their tranquillizing effects by acting on the same receptors.

In animals treated repeatedly with α-methyltyrosine, the tyrosine hydroxylase inhibitor (Nagatsu, Levitt & Udenfriend, 1964) which lowers tissue noradrenaline content (Spector, Sjoerdsma & Udenfriend, 1965), we found that the central actions of both reserpine and chlorpromazine were markedly enhanced. Since the pharmacological actions of asarone are in some ways comparable to those of reserpine and chlorpromazine we also examined the effect of asarone in animals treated with α-methyl-L-tyrosine.

Experimental

A solution of asarone for injection was prepared by dissolving it in a few drops of absolute ethanol and adding warm 3% polysorbate 80 to the required volume. α-Methyl-L-tyrosine was dissolved in the minimum quantity of 3N sodium hydroxide and the pH adjusted to 8 using 0·1N hydrochloric acid. Rats or mice were used according to which species was the more suitable for a particular experiment. All the injections were made intraperitoneally.

The albino mice used were of either sex and weighed between 20–25 g. They were of Hindustan Antibiotics strain.

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Noradrenaline content of whole brain of rats. Albino rats (CDRI strain) of either sex weighing between 100 and 150 g were given asarone (3 mg/kg) and after 45 min the animals were decapitated and the noradrenaline content of whole brain was estimated according to the method of Shore & Olin (1958). Animals treated with the solvent served as controls.

Effect of reserpine on the spontaneous motor activity of mice pretreated with asarone. A group of 20 mice was divided into 4 groups of 5 animals each. The first group was treated with reserpine (1 mg/kg). The second group was pretreated with asarone (3 mg/kg) followed 30 min later by reserpine (1 mg/kg). Since pretreatment with tetrabenazine prevents the action of reserpine (Quinn, Shore & Brodie, 1959), this drug was administered in a dose of 60 mg/kg to the third group of mice. 30 min later the animals were given reserpine (1 mg/kg). Solvent-treated animals served as controls. The four groups were observed for 6 hr and again 18–20 hr later, and compared. The degree of locomotor activity of groups of animals, their response to tactile stimulus, as well as the extent of ptosis in individual animals were scored and compared with those of the controls.

Effect of reserpine on the conditioned avoidance response of rats pretreated with asarone. Male albino rats (Haffkine strain) weighing between 100 and 125 g were trained to develop a conditioned avoidance response and an escape response. The technique is a minor modification of that of Cook & Weidley (1957) and is described in a previous communication (Dandiya & Sharma, 1962). Experiments were made between 9.00 and 12.00 hr at a room temperature of 24 $\pm 1°$.

Three groups of 10 trained rats each were treated with asarone (3 mg/kg), reserpine (1 mg/kg), and tetrabenazine (80 mg/kg) respectively. Another two groups of trained rats were pretreated with either asarone (3 mg/kg) or tetrabenazine (80 mg/kg), both were then given reserpine (1 mg/kg) 30 min later. 24 hr later the animals were examined for changes in their trained responses.

Effect of asarone on the rectal temperature of α-methyltyrosine-treated mice. Four groups of mice of 10 animals each were used. One group was treated with the solvent, another with asarone (1 mg/kg), a third with α-methyltyrosine followed by the solvent, and the fourth with α-methyltyrosine followed by asarone (1 mg/kg). α-Methyltyrosine was administered in three doses of 80 mg/kg each, the first dose was given 24 hr before, the second dose 18 hr before, the third dose 4 hr before either asarone or the solvent. Rectal temperature was recorded electronically in all the groups of mice immediately before the administration of asarone or solvent. The temperature recordings were again made 30 min after asarone or solvent administration, then at 1 hr intervals for the next 4 hr and again 16 hr later.

Effect of asarone on pentobarbitone-induced hypnosis of α-methyltyrosine-treated mice. The method adopted was similar to that of Dandiya & Cullumbine (1959). Groups of mice were treated with three doses of α-methyltyrosine according to the schedule mentioned above and 4 hr

after the last dose either the solvent or asarone (3 mg/kg) was given, 45 min later pentobarbitone sodium (40 mg/kg) was given.

Effect of asarone on the conditioned avoidance response of α-methyltyrosine-treated rats. Groups of rats trained for conditioned avoidance and escape responses were treated with α-methyltyrosine as mentioned previously. Asarone (3 mg/kg) was given 4 hr after the last dose of α-methyltyrosine. The animals were then examined for the loss of conditioned avoidance or escape responses at 1 hr intervals for 4 hr and then 24 hr later. The effect was compared with those obtained in control animals treated only with asarone (3 mg/kg) and also with rats receiving only α-methyltyrosine.

Results

Effect of asarone on the noradrenaline content of whole brain of rats. Asarone did not alter the noradrenaline content of whole brain of rats. The mean value (±s.e.) obtained in 6 solvent-treated animals was 0·508 ±0·088, and this was similar to that from 6 asarone-treated animals (0·521 ±0·090).

Effect of reserpine on the spontaneous motor activity and ptosis of mice pretreated with either asarone or tetrabenazine. Mice treated with either asarone or tetrabenazine were found to be sedated as seen by the reduced spontaneous activity and the degree of ptosis. Asarone caused a greater degree of sedation than tetrabenazine. The effect of these drugs lasted for 4–6 hr. The intensity of sedation observed in reserpine-treated animals was comparable to that of asarone, but the effect could be observed after 20 hr. When observed 18–20 hr after treatment with reserpine, animals which received asarone followed by reserpine showed an intensity of sedation and ptosis similar to the group treated with reserpine only. On the contrary, in animals treated with tetrabenazine and then reserpine, neither sedation nor ptosis was observed and responses resembled those of the solvent-treated controls. Thus whereas tetrabenazine blocked the effect of reserpine, asarone was ineffective.

Effect of reserpine on the conditioned avoidance response or escape response of rats pretreated with either asarone or tetrabenazine. The results are given in Table 1. The effect of asarone or tetrabenazine on

TABLE 1. EFFECT OF PRETREATMENT WITH ASARONE OR TETRABENAZINE ON THE CONDITIONED AVOIDANCE RESPONSE (CAR) AND ESCAPE RESPONSE (ER) BLOCKING EFFECT OF RESERPINE

Drug	No. of trained rats	20 hr after drug No. of animals showing blockade of		No. animals showing drug effect/total no. animals employed
		CAR	ER	
Control (untreated)	30	0	0	—
Reserpine	10	6	1	7/10
Asarone	10	0	0	0/10
Tetrabenazine	10	0	0	0/10
Asarone + reserpine	10	6	2	8/10
Tetrabenazine + reserpine ..	10	1	0	1/10

the conditioned avoidance response of trained rats did not persist for more than 4 hr and was not observed when the animals were examined 18–20 hr after drug treatment. On the other hand, in animals treated with reserpine only, a blockade of the conditioned avoidance response was observed in 60% of the animals. Almost similar results were obtained in rats receiving the asarone-reserpine combination. In contrast to this, tetrabenazine effectively blocked the action of reserpine.

Effect of α-methyltyrosine treatment on the pharmacological actions of asarone. The solvents employed for dissolving asarone or α-methyltyrosine did not influence the results of any of these experiments.

(*a*) Rectal temperature of mice: treatment of animals with α-methyltyrosine alone did not alter the rectal temperature of mice but, in α-methyltyrosine-pretreated animals, asarone brought about a more intense and prolonged fall in body temperature than observed with asarone alone.

(*b*) Pentobarbitone-induced hypnosis in mice: α-methyltyrosine pretreatment did not cause any change in the pentobarbitone-induced hypnosis in mice (Table 2). Pretreatment of the animals with asarone caused a prolongation in their sleeping time ($P < 0.02$) and this effect was more pronounced in α-methyltyrosine-pretreated animals ($P < 0.001$).

TABLE 2. EFFECT OF ASARONE ON THE PENTOBARBITONE-INDUCED HYPNOSIS OF α-METHYL-L-TYROSINE (AMT) TREATED MICE

Drug	No. of animals	Sleeping time in min ± s.e.
Pentobarbitone sodium	20	62·0 ± 12·0
AMT + pentobarbitone sodium	10	71·0 ± 8·8
Asarone + pentobarbitone sodium	10	132·8 ± 18·6 ($P < 0.02$)*
AMT + asarone + pentobarbitone sodium	10	212·0 ± 11·0 ($P < 0.001$)†

* P value was calculated by comparing with the group receiving pentobarbitone sodium only.

† P value was calculated by comparing with groups treated with both AMT and pentobarbitone sodium.

(*c*) Conditioned avoidance response of trained rats: Repeated administration of α-methyltyrosine to rats trained for conditioned avoidance response did not influence their acquired behaviour (Table 3). In a dose of 1 mg/kg asarone blocked this response in 30% of the animals, but the

TABLE 3. EFFECT OF ASARONE ON THE CONDITIONED AVOIDANCE RESPONSE (CAR) AND THE ESCAPE RESPONSE (ER) OF TRAINED RATS PRETREATED WITH α-METHYL-L-TYROSINE (AMT)

Drug	Hr after which the animals were examined after treatment with the last dose of		No. of animals	CAR lost	ER lost
	AMT	Asarone			
Control (untreated)	—	—	30	0	0
AMT	6	—	20	0	0
Asarone	—	2	10	3	1
AMT + asarone	6	2	10	4	9 ($P < 0.01$)

P value was calculated using χ^2 test by comparing with the results obtained in animals treated with asarone only.

escape response was not significantly influenced. This effect of asarone was markedly enhanced in animals treated with α-methyltyrosine, 90% losing their escape response as well. The effect of asarone on conditioned avoidance response lasted only for 5 hr, but of animals which received α-methyltyrosine and asarone, 40% showed a blocking effect on conditioned avoidance response even when examined after 24 hr.

Discussion

Previous studies in rats have shown that the tranquillizing effect of asarone is not mediated through changes in the brain 5-HT level (Dandiya & Menon, 1963), and unlike reserpine, which produces a stimulant effect in iproniazid-treated animals (Shore & Brodie, 1957), asarone causes sedation in mice treated with iproniazid (Dandiya & Menon, 1964). Since the structure of asarone resembles parts of the reserpine molecule, we wondered whether these two drugs acted on the same receptors to bring about the tranquillizing effect. Quinn, Shore & Brodie (1959) have shown that tetrabenazine, which also acts by lowering catecholamines, if administered before reserpine is capable of blocking its effect, thereby showing that both drugs act on the same receptors. We have found that asarone treatment failed to block the effect of reserpine in rats. More conclusive evidence was obtained in rats trained for the conditioned avoidance response. Whereas pretreatment with tetrabenazine blocked the reserpine effect, in animals which received asarone before reserpine, the blocking effect on the conditioned avoidance response of reserpine was unaffected, proving that the site of action of reserpine and asarone are different. Moreover, unlike reserpine, asarone failed to lower brain noradrenaline.

α-Methyltyrosine, which lowers brain noradrenaline content without affecting the 5-HT level (Spector & others, 1965), enhances some important actions of reserpine and chlorpromazine. It has been suggested that the enhancement is related to the lowered noradrenaline content of the hypothalamus (Menon, Bapna & Dandiya, 1966). The dosage schedule of α-methyltyrosine we have used caused a lowering of brain noradrenaline of rats and mice by 65%. Since noradrenaline has been suggested as a possible neurotransmitter of the ergotropic system of the hypothalamus (Brodie, Spector & Shore, 1959) a reduction in the content of this amine by reserpine and chlorpromazine would allow a further predominance of the trophotropic system in which 5-HT has been suggested as the neurotransmitter. Our present findings show that all three pharmacological actions of asarone were more marked in α-methyltyrosine-treated animals. The explanation for the effect of reserpine and chlorpromazine could be applied also to asarone. The antagonism of asarone to the stimulation produced by the ergotropic system stimulants like lysergic acid diethylamide and amphetamine (Dandiya & Menon, 1964) supports this view. But the ineffectiveness of asarone in lowering either brain 5-HT (Dandiya & Menon, 1963) or noradrenaline suggests that its mechanism of action is different from that of reserpine; it is probably more closely related to

that of chlorpromazine. The fact that both chlorpromazine and asarone effectively antagonize the central excitatory effects of mice treated with iproniazid before reserpine (Dandiya & Menon, unpublished observation) is additional evidence for this possibility.

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A polarographic limit test for sulphoxide in chlorpromazine

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Twin cell subtractive cathode ray polarography can be used as a quantitative limit test for from 0·5% to 2·0% of chlorpromazine sulphoxide in chlorpromazine. 0·05% of the impurity is detectable by derivative polarography.

EXISTING chromatographic limit tests for sulphoxide in phenothiazine drugs are time consuming, imprecise and require great care in order to avoid oxidation and photodegradation of the parent compound. A polarographic method is suggested for chlorpromazine sulphoxide in chlorpromazine, based on the fact that the sulphoxide gives a cathodic wave in 0·5N hydrochloric acid whereas the drug does not (Porter & Beresford, 1966).

The characteristic direct sulphoxide wave is shifted to a more negative peak voltage in the presence of a large excess of chlorpromazine, and appears as an unreadable shoulder on the final current rise. Derivative operation, while giving a rough quantitative estimation of sulphoxide content, does not resolve the wave completely. Twin cell subtractive operation gives complete resolution of the wave given by the sulphoxide, enabling concentrations of it from 0·5 μg/ml to 2·0 μg/ml to be determined in the presence of 100 μg/ml of chlorpromazine.

Experimental and results

APPARATUS

A Davis differential cathode ray polarograph was used (A1660, Southern Analytical Ltd.) with mercury pool anodes, and capillaries balanced by the normal procedure. All solutions were prepared and examined in subdued daylight, used within 2 hr of preparation and flushed with nitrogen (10 min) before polarographing.

CHLORPROMAZINE AND CHLORPROMAZINE SULPHOXIDE

Both are available as pure bases and solutions were prepared (chlorpromazine 500 μg/ml, chlorpromazine sulphoxide 25 μg/ml) in 0·005N hydrochloric acid. The chlorpromazine was examined polarographically in 0·5N hydrochloric acid at a concentration of 100 μg/ml using the 50 msec derivative circuit and maximum instrument sensitivity. No cathodic wave was seen (Fig. 1A). The sulphoxide (0·5 μg/ml) gave the expected wave under the same conditions (Fig. 1B).

Inclusion of 0·2 μg/ml of the sulphoxide with the chlorpromazine (100 μg/ml) produced a distinct wave using the 50 msec derivative circuit, maximum sensitivity and a start potential of −0·6 V (Fig. 2A). The limit of detection for sulphoxide under these conditions was not greater than 0·05 μg/ml (Fig. 2B).

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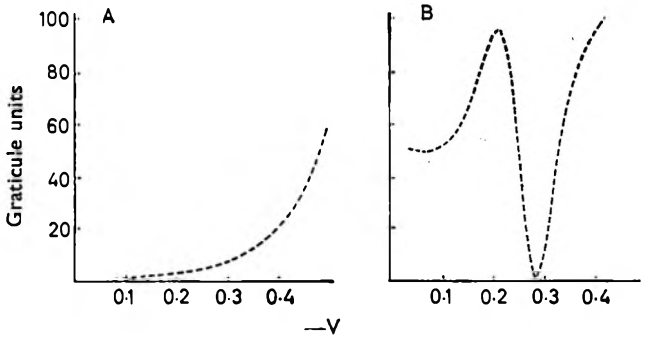

FIG. 1. Polarogram of (A) chlorpromazine (100 μg/ml), (B) chlorpromazine sulphoxide (0·5 μg/ml); 50 msec derivative, sensitivity maximum, start potential −0·6 V, supporting electrolyte 0·5 N hydrochloric acid.

PROCEDURE FOR CALIBRATION

Twin cell operation was carried out, the reference cell (cell 2) containing the base (100 μg/ml) in 0·5N hydrochloric acid. The test cell (cell 1) contained the base (100 μg/ml) and successive concentrations of the sulphoxide between 0·5 μg/ml and 2·0 μg/ml in 0·5N hydrochloric acid.

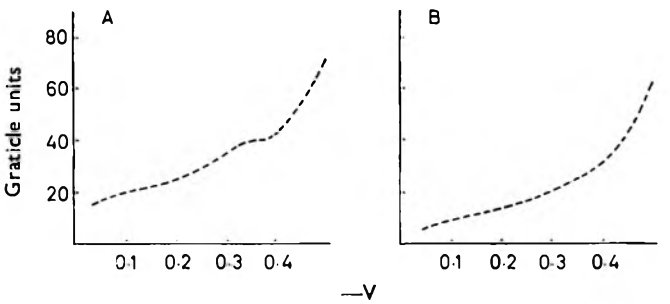

FIG. 2. Polarogram of chlorpromazine sulphoxide (A. 0·2; B. 0·05 μg/ml) in the presence of chlorpromazine (100 μg/ml); 50 msec derivative, sensitivity maximum, start potential −0·6 V, supporting electrolyte 0·5 N hydrochloric acid.

With a start potential of −0·6 V the subtractive wave peak occurred at about −0·93 V and a peak current/sulphoxide concentration graph was constructed, using the mean of five determinations at each sulphoxide concentration level (Table 1).

TABLE 1. CALIBRATION FOR TWIN CELL POLAROGRAPHY OF CHLORPROMAZINE SULPHOXIDE IN THE PRESENCE OF CHLORPROMAZINE (100 μG/ML)

Sulphoxide concentration (μg/ml)	Peak current (graticule units)		Sensitivity	Peak current at maximum sensitivity
	Mean	Standard deviation		
0·5	56	2·4	0·4 max.	140
1·0	67	1·3	0·25 ,,	268
1·5	70	1·1	0·16 ,,	420
2·0	92	1·0	0·16 ,,	552

Polarograms for cells 1 and 2 and the subtractive wave for 0·5 μg/ml sulphoxide as shown in Fig. 3A-C, respectively.

LIMIT TEST FOR SULPHOXIDE IN CHLORPROMAZINE HYDROCHLORIDE

A solution is prepared of the sample under test (112 μg/ml) in 0·5N hydrochloric acid. If no wave is seen on 50 msec derivative operation at maximum sensitivity, the sample does not contain more than 0·1% sulphoxide. If a wave is seen in the −0·93 V region, twin cell operation is carried out, using as reference either pure chlorpromazine base (100 μg/ml) or the pure hydrochloride (112 μg/ml) in 0·5N hydrochloric acid.

The peak current of the resolved wave should not be greater than that of a standard solution containing 100 μg/ml of pure chlorpromazine base (or an equivalent amount of pure hydrochloride) and the required concentration of the sulphoxide. A fresh reference solution is used for each determination.

Application of the test to a prepared solid mixture of sulphoxide (1%) in chlorpromazine, gave a peak current reading of 280 graticule units at maximum sensitivity.

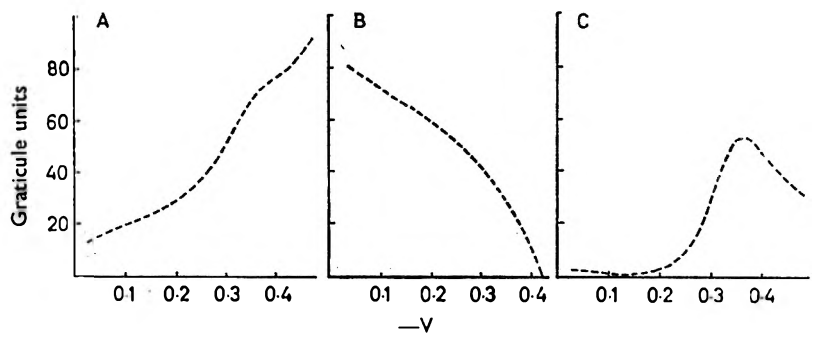

FIG. 3. A. Direct polarogram of chlorpromazine sulphoxide (0·5 μg/ml) and chlorpromazine (100 μg/ml). B. Direct polarogram (inverted) of chlorpromazine (100 μg/ml). C. Subtractive sulphoxide wave (0·5 μg/ml), sensitivity 0·4 maximum, start potential −0·6 V, supporting electrolyte 0·5N hydrochloric acid.

Discussion

The method is rapid (½ hr), accurate, sensitive, and has been applied successfully to promethazine and fluphenazine. A wave due to 0·2 μg/ml chlorpromazine sulphoxide can readily be resolved.

The method will not, of course, distinguish between the sulphoxide and any other reducible decomposition product which generates an inseparable wave, but this consideration is not of prime importance as the total quantity of such decomposition products may be expressed in terms of sulphoxide.

No polarographically detectable deterioration occurred with either chlorpromazine or its sulphoxide in 0·5N hydrochloric acid under the conditions employed. Solutions exposed to normal laboratory bench

daylight conditions for 8 hr developed in the case of chlorpromazine a wave indistinguishable from the sulphoxide wave, whereas sulphoxide solutions under these conditions showed a diminution in height of the characteristic wave. Solutions kept in the dark showed no deterioration for some days.

Acknowledgement. The chlorpromazine and chlorpromazine sulphoxide samples were kindly supplied by Messrs. May and Baker Ltd., Dagenham.

Reference

Porter, G. S. & Beresford, J. (1966). *J. Pharm. Pharmac.*, **18**, 223–227.

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The effect of succinylcholine on the isolated retractor bulbi muscle of the cat*

PAUL BACH-Y-RITA, JOSEPH V. LEVY, AND ANTOINETTE STEINACKER

The retractor bulbi muscle of the cat does not contain multi-innervated slow fibres, and succinylcholine (suxamethonium), 1–1000 μg/ml, causes no contracture of the isolated muscle. However, succinylcholine in a concentration of 1 μg/ml causes a contracture of the isolated inferior oblique and rectus muscles of the cat eye.

VARIOUS drugs cause a contracture of extraocular muscles; these drugs include acetylcholine (Duke-Elder & Duke-Elder, 1931) and depolarizing neuromuscular blocking drugs such as succinylcholine (Hofman & Lembeck, 1952, Dillon, Sabawala, Taylor & Gunter, 1957). In this respect, the extraocular muscles resemble denervated skeletal muscles (Brown, 1937).

The rectus and oblique muscles of the cat eye contain both singly and multi-innervated muscle fibres (Hess & Pilar, 1963, Peachey, 1967); the former, and at least some of the latter, are twitch fibres (Bach-y-Rita & Ito, 1966). The multi-innervated fibres respond to succinylcholine by contracture (Bach-y-Rita & Ito, 1966). Histological (Alvarado, Steinacker & Bach-y-Rita, 1967), physiological and pharmacological studies *in vivo* (Bach-y-Rita & Ito, 1965) have shown that the retractor bulbi muscle of the cat does not contain slow fibres like those found in other extraocular muscles; the fibres resemble typical skeletal muscle twitch fibres.

In the *in vivo* preparation, with the eyeball enucleated, succinylcholine-induced contracture of the rectus and oblique muscles may produce an apparent shortening of the retractor bulbi muscle, which can be prevented by tying the tendons of the rectus and oblique muscles to prevent the muscles shortening. We have now compared the effect of succinylcholine on the isolated retractor bulbi muscle, which consists of singly innervated twitch fibres only, with the effect on isolated rectus and oblique muscles, which consist of both singly and multi-innervated muscle fibres.

Methods

Nine retractor bulbi muscle slips, 3 inferior oblique, 1 superior rectus and 1 lateral rectus muscle were studied. The muscles were obtained from cats anaesthetized with phenobarbitone. A portion of the sclera, including the muscle insertion, was cut from the eyball. The eyeball was removed from the cat and the muscle dissected free as close to the origin as possible. The muscle, attached by the portion of sclera to the mounting assembly, was suspended in a 50 ml bath containing modified Krebs-Henseleit solution at 37°, and gassed with oxygen 95% and carbon dioxide 5%. An average tension of 1·0 g was applied to the muscle. The method

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of recording isometric contractions and the formula of the modified Krebs-Henseleit solution are described fully elsewhere (Levy & Richards, 1965). The concentration of succinylcholine chloride is expressed in μg/ml.

Results

Succinylcholine (1 μg/ml) caused an immediate contracture of the inferior oblique and the rectus muscles. Larger contractures are produced by increasing the concentration of succinylcholine by 10 and 20 μg/ml (Fig. 1a). Succinylcholine in concentrations of 1–1000 μg/ml caused no contracture of the retractor bulbi muscle (Fig. 1b). However, succinylcholine in a concentration of 100 μg/ml often caused a brief contraction of less than 40 mg tension for less than 2 sec. This response is similar in time course to that of the gastrocnemius muscle of the cat on administration of acetylcholine *in vivo* (Brown, Dale & Feldberg, 1936).

At the end of each experiment the muscle contracted to a direct electrical stimulus. This demonstrated its viability.

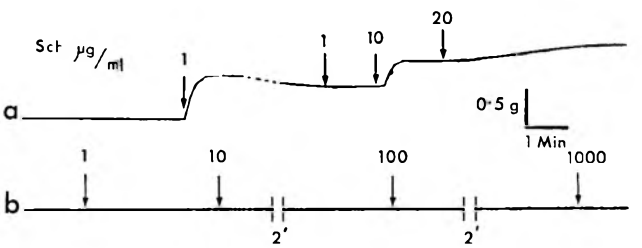

FIG. 1. Responses of isolated retractor bulbi and inferior oblique muscles of the cat to succinylcholine. Succinylcholine was added cumulatively to the bath at the arrows to increase the concentration, in μg/ml, in the bath by the amount indicated above these arrows. Contraction of a muscle is indicated by an upward deflection of the trace. Trace (a) shows the responses of the inferior oblique muscle and trace (b) those of the retractor bulbi muscle.

Discussion

Alvarado & others (1967) have shown that the retractor bulbi of the cat resembles normal skeletal muscle in being made up of singly innervated large twitch fibres. The rectus and oblique muscles, however, are made up of both singly and multi-innervated fibres. It would be expected that succinylcholine would cause a contracture of these muscles and not the isolated retractor bulbi muscle. The present experiments have confirmed this expectation.

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The accumulation of [^{3}H]noradrenaline in the adrenergic nerve fibres of reserpine-treated mice

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Mice, pretreated with reserpine, were given [^{3}H]noradrenaline (^{3}H-NA) intravenously and were killed 30 min later. Only small amounts of ^{3}H-NA were recovered from the hearts. The monoamine oxidase inhibitor nialamide in a dose of 10 mg/kg increased the ^{3}H-NA recovered from the hearts of reserpine-treated animals severalfold. In higher doses, however, this effect of nialamide was no longer observed. When ^{3}H-NA was replaced by [^{3}H]α-methylnoradrenaline, nialamide only reduced the amount of amine recovered from the hearts. The balance between the opposing actions of nialamide depended on dosage, time interval and temperature in a manner suggesting that the inhibitory influence was due to accumulation of endogenous amines in the adrenergic nerve fibres. Bretylium and harmaline also preserved ^{3}H-NA in reserpine-treated animals whereas iproniazid, pheniprazine, pargyline and tranylcypromine did not do this. Moreover, pargyline blocked the ability of nialamide to enhance ^{3}H-NA accumulation. The uptake of ^{3}H-NA observed after monoamine oxidase inhibition in the hearts of reserpine-treated mice was almost completely blocked by pretreatment with desipramine or protriptyline, potent blockers of amine uptake by adrenergic nerve fibres.

IN 1964, Hamberger, Malmfors, Norberg & Sachs, using a histochemical method, were able to demonstrate that the adrenergic neuron can take up and concentrate noradrenaline (NA) even after pretreatment with reserpine. Later this uptake mechanism was shown to be located at the level of the cell membrane, to be selectively blocked by cocaine, and to differ from the storage mechanism of the granules which is blocked by reserpine (Hillarp & Malmfors, 1964). These findings provided the first direct evidence for the dual amine uptake and storage mechanism of the adrenergic neuron postulated by Carlsson, Hillarp & Waldeck (1963). On the basis of experiments on the perfused rat heart, Muscholl (1963) suggested a reserpine-resistant uptake of NA into the cell. To achieve an accumulation of NA in the nerves of reserpine-treated animals it is necessary to block monoamine oxidase which otherwise will destroy the NA taken up, since reserpine excludes its protection by the amine-granules. This agrees with the findings of Kopin, Hertting & Gordon (1962) that monoamine oxidase plays a major role in the metabolism of NA in the hearts of reserpine-treated rats.

The reserpine-resistant uptake of NA has been further examined by Malmfors (1965), Carlsson & Waldeck (1966a) and, *in vitro*, by Hamberger & Masucka (1965). At first when we tried to reproduce the histochemical findings biochemically we ran into difficulties, the uptake being relatively small (Andén, Carlsson & Waldeck, 1963). Later we found that the dose of the monoamine oxidase inhibitor was critical. Moreover, successful results were achieved only with certain monoamine oxidase inhibitors; others reduced the accumulation and antagonized the effect of inhibitors which increased accumulation.

It therefore seemed to be important to determine the optimal conditions under which NA can accumulate in the adrenergic nerves of reserpine-treated animals.

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Experimental

MATERIALS

Commercially available (±)-[7-^{3}H]noradrenaline* (^{3}H-NA) with a specific activity of about 6 c/m-mole was used. α-Methyl[7,8-^{3}H]noradrenaline** (^{3}H-α-Me-NA) with an approximate specific activity of 30 mc/m-mole was prepared in co-operation with the Research Laboratories of Hässle Ltd (Hallhagen, G. & Waldeck, B., unpublished). Drugs used were: bretylium tosylate (Burroughs Wellcome & Co.); desipramine hydrochloride (Geigy); harmaline; iproniazid phosphate (Roche); nialamide (Swedish Pfizer); pargyline (Dr. G. M. Everett, Abbott Laboratories); pheniprazine hydrochloride (Draco); protriptyline hydrochloride (Dr. C. A. Stone, Merck Institute for Therapeutic Research); reserpine (Swedish Ciba); tranylcypromine sulphate (Smith, Kline & French).

METHODS

When not otherwise stated the experiments were made at room temperature (22–24°). Mice, divided at random into groups of six, were given reserpine (10 mg/kg i.p.) 6 hr, and a monoamine oxidase inhibitor in various doses and at different times, before the intravenous injection of 1 μg/kg ^{3}H-NA or 20 μg/kg ^{3}H-α-Me-NA. Thirty min later the animals were killed, and the hearts removed and extracted in 0·4N perchloric acid. The extracts were chromatographed on Dowex 50 W X4 ion-exchange resin. ^{3}H-NA and ^{3}H-α-Me-NA were determined in the eluate by liquid scintillation counting (for details see Carlsson & Waldeck, 1963; and unpublished).

Results

Nialamide in various doses was given to reserpine-treated mice 2 hr before ^{3}H-NA or ^{3}H-α-Me-NA. After 30 min the animals were killed and their hearts were analysed for ^{3}H-NA and ^{3}H-α-Me-NA, respectively. In the absence of a monoamine oxidase-inhibitor, reserpine caused a pronounced reduction of the amount of ^{3}H-NA recovered (Fig. 1). With increasing doses of nialamide the amount of ^{3}H-NA at first increased, reaching a maximum at about 10 mg/kg; it then decreased so that, at a dose of 100 mg/kg of nialamide, ^{3}H-NA was at about the same level as when reserpine had been given alone. The concentration of ^{3}H-NA observed after 10 mg/kg of nialamide was about 0·9 ng/g which is about one half of the value observed in normal animals at this time interval (compare Table 1). When the experiment was made at 30° the ^{3}H-NA levels were much lower. Even here a maximum was obtained after about 10 mg/kg of nialamide.

In reserpine-treated animals, 49 ng/g of ^{3}H-α-Me-NA was found in the heart 30 min after the administration. This is about the same amount as in animals not pretreated with reserpine (Carlsson, A. & Waldeck, B., unpublished). Nialamide had only an inhibitory influence on the accumulation

* 2-Amino-1-(3,4-dihydroxyphenyl)-[1-^{3}H] ethanol.
** 2-Amino-1-(3,4-dihydroxyphenyl)-[1,2-^{3}H] propanol.

of ^{3}H-α-Me-NA. With increasing doses of nialamide, the amount of ^{3}H-α-Me-NA decreased continuously so that at a dose of 100 mg/kg of nialamide only 12 ng/g could be detected. Between 10 and 100 mg/kg of nialamide, the ^{3}H-NA and ^{3}H-α-Me-NA curves (at 22–24°) took a similar course.

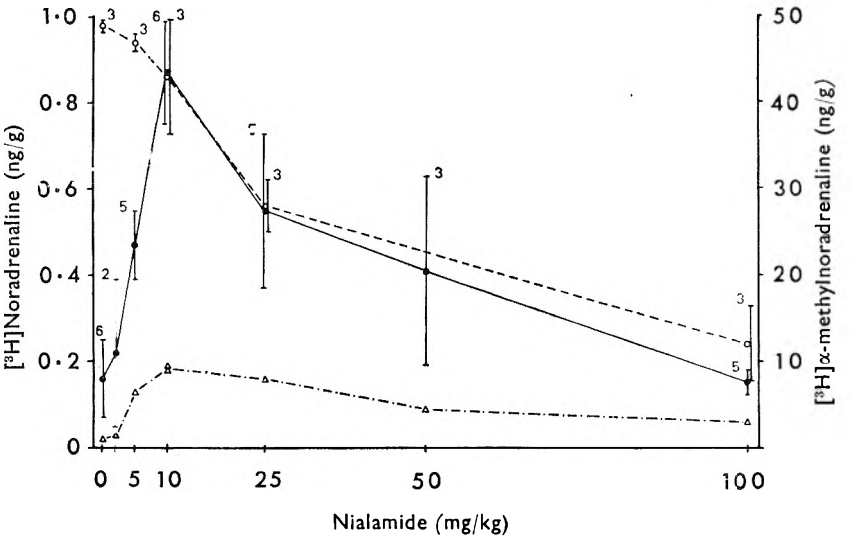

FIG. 1. Effect of nialamide on the accumulation of [^{3}H]noradrenaline and [^{3}H]α-methylnoradrenaline in the hearts of reserpine-treated mice. Reserpine (10 mg/kg) was given to mice i.p. 6 hr, and nialamide in various doses i.p. 2 hr, before intravenous [^{3}H]noradrenaline (1 μg/kg) or [^{3}H]α-methylnoradrenaline (20 μg/kg). The animals were killed 30 min later and the amine levels in the hearts were determined. Shown are the means ± s.e.m. with the number of experimental groups indicated at each point. However, the symbols of the curve obtained at an ambient temperature of 30° represent single values. Each experimental group consists of 6 animals. △----△, +30°, [^{3}H]noradrenaline. ●——●, +22–24°, [^{3}H]noradrenaline. ○----○, +22–24°, [^{3}H]α-methylnoradrenaline.

A number of monoamine oxidase inhibitors and bretylium were tested for their ability to preserve ^{3}H-NA taken up in the hearts of reserpine-treated mice (Table 1). Of these, only bretylium, harmaline and nialamide proved effective. When nialamide in a high dose (100 mg/kg) was given a short time (15 min) before ^{3}H-NA it appeared to be almost as efficient as 10 mg/kg given 2 hr before (compare Fig. 1).

In another experiment nialamide or pargyline, or a combination of both, was given to reserpine-treated mice before ^{3}H-NA. Pargyline proved inefficient in restoring ^{3}H-NA uptake in reserpine-treated animals, and moreover, blocked the positive effect of nialamide (Fig. 2). As the dose of pargyline used could be regarded as relatively high, a second experiment was designed using a smaller dose (10 mg/kg) given intravenously at various times before or after ^{3}H-NA (Fig. 3). Given 60 or 5 min before, pargyline inhibited the accumulation of ^{3}H-NA by about 2/3. When given 5 or 15 min after ^{3}H-NA, however, pargyline seemed to be less efficient in this respect.

Desipramine or protriptyline, efficient and specific blockers of the amine uptake at the level of the cell membrane of the adrenergic nerve fibre, were given a short time before ^{3}H-NA to mice pretreated with reserpine and nialamide. This resulted in a reduction of ^{3}H-NA uptake to the level observed in animals pretreated with reserpine only (Fig. 4).

TABLE 1. EFFECT OF SOME MONOAMINE OXIDASE INHIBITORS AND BRETYLIUM ON THE ACCUMULATION OF [^{3}H]NORADRENALINE (^{3}H-NA) IN THE HEARTS OF RESERPINE-TREATED MICE. Reserpine and a monoamine oxidase inhibitor were given at various times before i.v. injection of 1 μg/kg ^{3}H-NA. The animals were killed 30 min later and the levels of ^{3}H-NA in the hearts were estimated. Experiments made at an ambient temperature of 30° are indicated by an asterisk. The other experiments were made at 22–24°

Reserpine 10 mg/kg i.p. hr before	Inhibitor	Dose mg/kg	Hr before ^{3}H-NA	^{3}H-NA ng/g ± s.e.m.	n
None	None	—	—	1·90 ± 0·11	17
6	None	—	—	0·08 ± 0·01	11
6	None	—	—	0·03 ± 0·01*	2
6	Bretylium	10 i.v.	½	0·25 ± 0·04	4
6	Harmaline	10 i.p.	½	0·53 ± 0·03	3
6	Harmaline	20 i.p.	½	0·66 ± 0·24	4
6	Iproniazid	10 i.p.	2	0·10	1
6	Iproniazid	100 i.p.	2	0·12	1
18	Iproniazid	100 i.p.	2	0·05	1
6	Nialamide	100 i.p.	½	0·53 ± 0·11	4
18	Nialamide	100 i.p.	2	0·21 ± 0·15	2
6	Pargyline	10 i.p.	½	0·02*	1
6	Pargyline	100 i.p.	½	0·03*	1
6	Pargyline	10 i.p.	2	0·10 ± 0·01	2
6	Pargyline	10 i.p.	2	0·02*	1
6	Pargyline	100 i.p.	2	0·04*	1
18	Pargyline	100 i.p.	2	0·05	1
6	Pargyline	400 i.p.	2	0·05	1
6	Pheniprazine	10 i.p.	2	0·07	1
6	Tranylcypromine	10 i.v.	½	0·03	1
18	Tranylcypromine	10 i.v.	½	0·03	1

Discussion

The ability of the adrenergic neuron to retain NA depends largely on the amine-storing granules. If the granular storage mechanism is impaired by, for example, reserpine, NA will still be taken up by the amine transport mechanism at the level of the cell membrane (the "membrane pump") but as the monoamine oxidase is intact no accumulation of NA can occur. With a proper dose of a suitable monoamine oxidase inhibitor the NA taken up by the membrane pump will accumulate and stay in the neuron for some time.

We have shown that the conditions under which such an accumulation can occur are stringent. When nialamide was given 2 hr before the ^{3}H-NA, a relatively small dose (10 mg/kg) seemed to be most efficient in increasing the amount recovered. When the dose of nialamide was increased, this reduced the amount of ^{3}H-NA. This action could be even more clearly demonstrated when ^{3}H-NA was replaced by ^{3}H-α-Me-NA, an amine resistant to monoamine oxidase and thus being able to accumulate in the adrenergic neurons of reserpine-treated animals in the absence of a monoamine oxidase inhibitor. Nialamide at 100 mg/kg, a

dose which had little effect in preserving ^{3}H-NA when given 2 hr before it, seemed to have a good effect when given 15 min before ^{3}H-NA. This supports the view that the effect of nialamide in reducing the amount of amine recovered is a secondary phenomenon. It is possibly a consequence of monoamine oxidase inhibition; that is, blockade of the enzyme will result in accumulation of endogenous amines which may then compete with exogenous amines for uptake and binding mechanisms of the adrenergic nerve fibres. The ability of nialamide to preserve ^{3}H-NA in the hearts was less marked if hypothermia was prevented by keeping the reserpine-treated mice at an elevated ambient temperature.

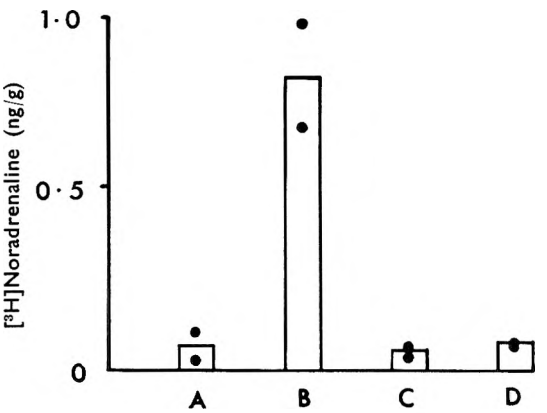

FIG. 2. Effect of pargyline on the accumulation of [^{3}H]noradrenaline in the hearts of mice. [^{3}H]Noradrenaline (1 μg/kg) was given i.v. to mice pretreated with reserpine, nialamide or pargyline as described below. The animals were killed 30 min later and the amine levels in the hearts were determined. Each point represents one experimental group consisting of six animals. A. Reserpine. B. Reserpine + nialamide. C. Reserpine — pargyline. D. Reserpine + nialamide + pargyline. Dose regimen: reserpine 10 mg/kg, i.p. 6 hr before [^{3}H]noradrenaline; nialamide 10 mg/kg, i.p. 2 hr before [^{3}H]noradrenaline; pargyline 100 mg/kg, i.p. 1 hr before [^{3}H]noradrenaline.

It has been observed (Carlsson, A. & Lindqvist, M., unpublished data, *see also* Carlsson, Dahlström, Fuxe & Lindquist, 1965) that the accumulation of monoamines in the brains of reserpine-treated mice brought about by nialamide or pargyline is much accelerated if hypothermia is prevented by keeping the animals at about 30°. If the inhibitory effect on accumulation by nialamide observed in the present experiments is due to accumulation of endogenous amines, it would be expected that this influence would be pronounced at an elevated temperature. Nialamide and pargyline have been shown previously to be capable of releasing [^{3}H]metaraminol from the hearts of mice not pretreated with reserpine (Carlsson & Waldeck, 1966b). Under these conditions it could be shown that inhibition of dopa decarboxylase blocked the releasing effect of the monoamine oxidase inhibitors (Carlsson, A., Lindqvist, M. & Waldeck, B., unpublished). The nature of the endogenous amines apparently involved here is obscure. It may be newly synthesized NA but other

amines such as octopamine cannot be excluded. Kakimoto & Armstrong (1962) have demonstrated the presence of octopamine in the rabbit heart after monoamine oxidase inhibition.

In view of the apparently subtle balance between the enhancing and antagonizing influences of monoamine oxidase inhibitors on ³H-NA accumulation in the heart it is not surprising that the scatter in some of the experiments was large.

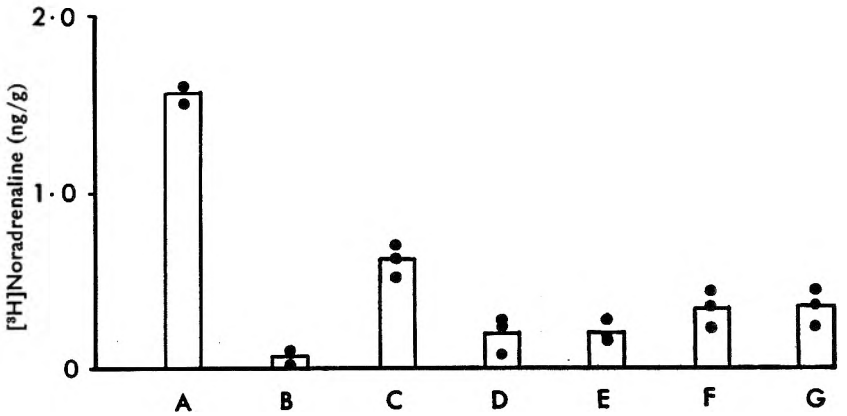

FIG. 3. Effect of pargyline on the accumulation of [³H]noradrenaline in the hearts of mice pretreated with reserpine and nialamide. Indicated are the times before or after the i.v. administration of [³H]noradrenaline (1 μg/kg). The animals were killed 30 min later and the amine levels were determined. Each point represents one experimental group consisting of six animals. A. Control. B. Reserpine. C. Reserpine + nialamide. D. Reserpine + nialamide + pargyline at −60 min, at −5 min (E), at +5 min (F), and at +15 min (G). Dose regimen: reserpine 10 mg/kg, i.p. 6 hr before [³H]noradrenaline; nialamide 10 mg/kg, i.v. 2 hr before [³H]noradrenaline; pargyline 10 mg/kg, i.v.

In experiments on reserpine-resistant uptake mechanisms it is important that the dose and time interval be adequately chosen; recovery of the granular function sets in early (Andén, Magnusson & Waldeck, 1964; Andén & Henning, 1966). In the present experiments a large dose of reserpine (10 mg/kg) and a relatively short interval corresponding to the maximum action of the drug have been used.

In addition to the monoamine oxidase inhibitors nialamide and harmaline, bretylium preserved ³H-NA in hearts of reserpine-treated mice. Bretylium has been observed to behave as an inhibitor of the enzyme under various experimental conditions (Malmfors, 1965; Furchgott, 1966; Carlsson, A. & Waldeck, B., unpublished). In fact, Jonason, J. (unpublished), has observed an inhibitory action of bretylium (5×10^{-4}M) on a monoamine oxidase preparation *in vitro*.

In the present experiments iproniazid, pargyline, tranylcypromine and pheniprazine proved inefficient in preserving ³H-NA in the hearts of reserpine-treated mice. A full explanation of this failure is not yet possible. The amphetamine-like structure and activity of tranylcypromine and pheniprazine may be important (cf. Carlsson & Waldeck, 1966b).

This inability to preserve ^{3}H-NA by certain inhibitors of the enzyme does not accord with the observations of Malmfors (1965) who found all the inhibitors tested enhanced accumulation of NA. But his observations were made on the iris of the rat and the histochemical method he used is very sensitive so that variations in NA concentration above a certain level may not have been detected. Pargyline not only proved inefficient in preserving ^{3}H-NA but even after small doses actually blocked the enhancing action of nialamide. It is doubtful whether this action is the result of an inhibition of the enzyme.

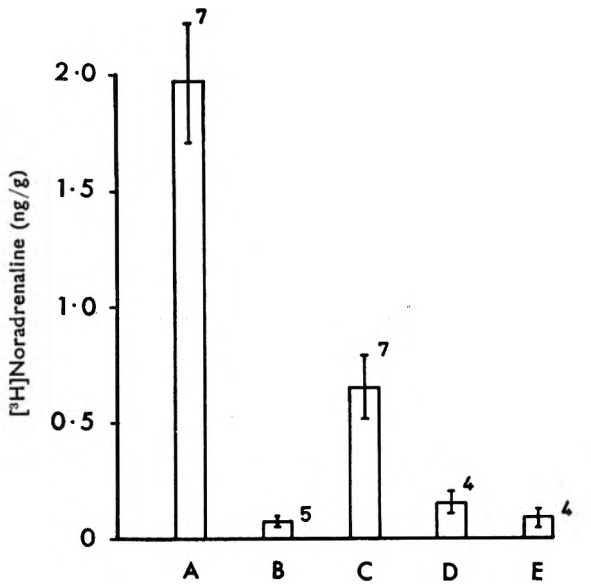

FIG. 4. Effect of desipramine and protriptyline on the accumulation of [^{3}H]noradrenaline in the hearts of mice pretreated with reserpine and nialamide. [^{3}H]Noradrenaline (1 μg/kg) was given i.v. to mice pretreated as described below. The animals were killed 30 min later and the level of [^{3}H]noradrenaline in the heart estimated. Shown are the means $\pm$ s.e.m. with the number of experimental groups indicated at each point. Each experimental group consists of six animals. A. Control. B. Reserpine. C. Reserpine + nialamide. D. Reserpine + nialamide + desipramine. E. Reserpine + nialamide + protriptyline. Dose regimen: reserpine 10 mg/kg, i.p. 6 hr before [^{3}N]noradrenaline; nialamide 10 mg/kg, i.p. 2 hr before [^{3}N]noradrenaline; disipramine 5 mg/kg, i.v. 5 min before noradrenaline; protriptyline 5 mg/kg, i.v. 5 min before [^{3}N]noradrenaline.

There is little doubt that the ^{3}H-NA determined is within the nerves. Histochemical work (Hamberger & others, 1964; Hillarp & Malmfors, 1964; Malmfors, 1965) clearly demonstrates that under conditions similar to those used by us exogenous NA accumulates in the adrenergic nerves. Further, potent and selective blockers of the "membrane pump" such as desipramine and protriptyline, when given before the test amine, effectively inhibit the accumulation of ^{3}H-NA in the hearts of reserpine-treated mice as well as the accumulation of NA in the adrenergic nerve fibres as observed histochemically. When the blocking agent is given after the

test amine, however, ^{3}H-NA already accumulated seems to be largely unaffected (Carlsson & Waldeck, 1966a).

The question arises whether the reserpine-resistant NA is free or particle bound. Recent work by Stitzel & Lundborg (cf. Lundborg & Waldeck, 1966) has shown that 30 min after the intravenous injection of ^{3}H-NA in mice pretreated with reserpine and nialamide as described here, as much as 15% of the ^{3}H-NA was found in the "particulate" fraction (calculated as per cent of the sum of particulate and supernatant fractions), that is, about one-third of the percentage observed in normal animals. These data indicate that at least part of the reserpine-resistant NA fraction is particle bound. Whether the particles in question are specific storage granules, microsomal particles, or fragments of the cell membrane is difficult to answer. It is interesting that, in the particulate fraction of the adrenal medulla, an amine uptake mechanism has been detected which does not require ATP and Mg^{2+} and is insensitive to reserpine (Lundborg 1966).

In the hearts of rats pretreated with reserpine 24 hr before the experiment, Iversen, Glowinski & Axelrod (1966) observed an uptake of ^{3}H-NA which occurred even when monoamine oxidase was intact and was not blocked by desipramine. Their observations were made only 5 min after the administration of the ^{3}H-NA. It seems probable that under such conditions most of the ^{3}H-NA is located outside the nerves. This would explain why the ^{3}H-NA uptake was unaffected by desipramine.

In a review, Costa, Boullin, Hammer & others (1966) have reached conclusions opposite to ours. They express the view that reserpine blocks amine uptake at the cell membrane level, whereas desipramine blocks uptake by amine granules. The data on which these conclusions are based, are only briefly reported in the article and do not seem to have been published.

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The presence of peroxidases in tragacanth

SIR,—In both the Pharmacopoeia Nordica (1963) and the Pharmacopoeia Belge (Suppl. 1940) the monograph for Tragacanth includes a test for the exclusion of acacia based on the well known fact that acacia contains peroxidases which give a distinctive blue colour in the presence of benzidine and hydrogen peroxide. Exporters from this country have complained that samples of genuine powdered tragacanth, containing no acacia, have been rejected on the basis of this test and accordingly I have examined a number of samples of tragacanth by this method. While there is a large number of grades, those suitable for pharmaceutical use are known as "ribbon" and "flake". The former grade consists of translucent, thin white ribbons and certainly fits the description given in the British Pharmacopoeia (1963); some samples of "flake" may also fit the description. Nine samples of ribbon and nine samples of flake (kindly supplied by Kimpton Brothers Ltd.), together with some samples from our own stocks, were tested using benzidine and hydrogen peroxide, as described in the British Pharmacopoeia (1963) under "Acacia", and using *o*-tolidine and hydrogen peroxide in exactly similar conditions. For each test the reagents were added to a cold mucilage and then to a boiled and cooled mucilage to act as a control. Only four samples (all ribbon) gave no blue colour; all other samples gave colours ranging from greenish-blue to dark royal blue. This shows that genuine samples of tragacanth may contain peroxidases and that a test based on their presence is useless for detecting adulteration with acacia.

Parallel tests for starch were also carried out by adding known quantities of N/50 iodine to the mucilage being tested. It was noted that those samples giving no blue colour for peroxidases were also free from starch and that, in the other samples, the intensity of the colour for peroxidases was paralleled by the intensity of the colour for starch. Furthermore the viscosity of the mucilages appeared to vary inversely with the intensity of these colours. Since tragacanth is formed in the plant by a process of "gummosis" from the cell walls and the starch contents (Gentry, 1957), it would seem that the finest grades, producing mucilages of high viscosity, are formed when the gummosis process completely converts all the starch into gum and, at the same time, causes the peroxidases to disappear. The ability to detect these changes in the finished gum by the use of benzidine (or *o*-tolidine), iodine and viscosity measurements may be of importance other than pharmaceutically, as Roe (1959) has drawn attention to the fact that ribbon tragacanth, in contrast to flake, inhibits mouse ascites tumour growth. Ribbon gum appears to attach itself to the ascites tumour cell, causing damage to it (Galbraith, Mayhew & Roe, 1962) and producing mitotic inhibition and cytostasis. Heat de-activated tragacanth and karaya gum were shown to have no such effects (Mayhew & Roe, 1964, 1965).

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Chlorpromazine-lysergic acid diethylamide antagonism

SIR,—Lysergic acid diethylamide (LSD) produces an increase in the 5-hydroxytryptamine (5-HT) content of rat brain (Freedman, 1961; Freedman & Giarman, 1962), which may be related to many of its central effects like tremors, excitement and hallucinations (Udenfriend, Weissbach & Bogdanski, 1957; Giarman & Freedman, 1965). It is also known that chlorpromazine antagonizes the LSD-induced behavioural effects in man and animals (Ray & Marrazzi, 1960) and the EEG changes in dogs (Djahanguiri & Guiti, 1966). We therefore examined the effect of chlorpromazine on the LSD-induced increase in the brain 5-HT.

Adult albino rats, 100–150 g, were injected intraperitoneally with LSD (0·25 mg/kg) and chlorpromazine (0·15 mg/kg). In one group of rats, LSD and chlorpromazine were given simultaneously; the second group had LSD followed by chlorpromazine after 10 min; a third group were given chlorpromazine 10 min before the LSD. Control animals had normal saline. The rats were killed ½ hr after the administration of drugs and the brain (excluding the olfactory lobe, cerebellum and pituitary glands) rapidly removed. The 5-HT was extracted by the method of Amin, Crawford & Gaddum (1954) and assayed on the oestrous uterus of the rat (Parratt & West, 1957).

TABLE 1. EFFECT OF CHLORPROMAZINE ON LSD-INDUCED INCREASE IN THE 5-HT CONTENT OF RAT BRAIN

Drugs	Dose mg/kg	Time interval in hr	No. of rats	Brain 5-HT content in µg/g ± s.e.	Probability P
Control	—	½	10	0·38	—
LSD	0·25	½	9	0·48 ± 0·03	<0·01
Chlorpromazine	0·15	½	7	0·37 ± 0·05	<0·05
LSD + Chlorpromazine	0·25 + 0·15	½	6	0·47 ± 0·006	<0·01
Chlorpromazine, followed by LSD (after 10 min)	0·15 + 0·25	½	6	0·45 ± 0·04	<0·01
LSD followed by Chlorpromazine (after 10 min)	0·25 + 0·15	½	8	0·46 ± 0·07	<0·05

The results (Table 1) show that pre-, simultaneous or post-administration of chlorpromazine does not modify the LSD-induced increase in brain 5-HT, even though according to Ray & Marrazzi (1960) it does antagonize the behavioural effects of the hallucinogen. This finding is in agreement with Hess & Doepfner (1961) who, pretreating rats with a monoamine oxidase inhibitor and then giving tryptophan, concluded that it was possible to produce alterations in brain 5-HT levels without corresponding changes in behaviour.

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Polyhedral emulsion particles

SIR,—In 1965, irregular polyhedral particles were reported in a semi-solid emulsion system (Groves & Scarlett, 1965) and it was suggested that they may have arisen in areas of localized close packing within the system which produced distortion of the molten oil phase droplets at elevated temperatures, the shape being retained when the droplets cooled and solidified. However, the fact that close packing could occur in a system with a disperse ratio of 0·225 was of interest since this would not be anticipated until the concentration approached a value of approximately 0·74, the theoretical limit for a system consisting of equal diameter spheres.

We have now examined systems similar to the emulsion system previously investigated and consisting of an oil phase of equal parts by weight of cetostearyl alcohol and liquid paraffin dispersed in 0·5% w/w aqueous cetrimide solutions. A series of dispersions containing different amounts of the oil phase were prepared under standardized conditions. The molten oil phase at 70° was added to the aqueous phase at the same temperature and stirred with a high speed laboratory stirrer (Silverson, fitted with homogenizer head) until the mixture had cooled to 30°, when the stirrer was switched off and cooling allowed to proceed undisturbed to room temperature.

Samples were withdrawn and particle size distributions measured with a Coulter Counter Model A (Industrial) fitted with a 70 μ orifice tube. Results are summarized in Table 1.

TABLE 1. PARTICLE SIZE DISTRIBUTIONS OF THE DISPERSIONS PREPARED WITH DIFFERENT DISPERSE PHASE RATIOS. MEASURED WITH A COULTER COUNTER MODEL A (INDUSTRIAL) FITTED WITH A 70 μ ORIFICE TUBE (GROVES, 1966)

Dispersion phase ratio (weight)	Particle size distribution parameters[1]	
	D50 (μ)	Standard deviation
0·01[2]	2·75	3·20
0·05	3·40	2·03
0·10	5·30	1·89
0·15	5·00	1·90
0·20	4·65	1·87
0·30	4·85	2·06
0·40	8·30	1·92
0·50	8·50	2·00
0·55[3]	8·00	2·21

[1] From a logarithmic probability plot of the cumulative weight percent distribution.
[2] The data on this sample is unreliable since microscope examination showed that most particles were below the limit of detection of the Counter under the conditions of measurement.
[3] Attempts to prepare systems more concentrated than 0·55 were not successful.

Using a photomicrographic technique, at least 1,000 particles from each sample were classified visually as either spherical or non-spherical and the results are shown in Fig. 1.

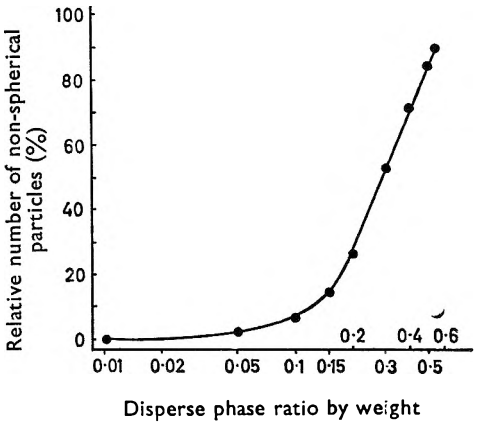

FIG. 1. The relative number of non-spherical particles in dispersions of different disperse phase ratio.

Although the particle size analysis of the most dilute system is not reliable since most of the particles appear to be below the discrimination of the Coulter Counter under the conditions of measurement, it will be seen from Table 1 that there is a general tendency for the particles to increase in size as the systems become more concentrated. This was also observed visually. In addition, the standard deviations of the size distributions remain relatively constant. However, the relative number of non-spherical particles shows a considerable increase above a disperse phase ratio of about 0·1. This latter effect supports the earlier contention that the polyhedral particles are unlikely to be due to crystallization of the high-melting point cetostearyl alcohol (Axon, 1957).

Viscosity measurements using a Ferranti-Shirley cone-plate viscometer indicate that the dilute systems behave as Newtonian fluids. Systems with disperse phase ratios in excess of 0·1 exhibit pseudoplastic flow and hysteresis, the amount of hysteresis increasing with increasing concentration. This also suggests that interparticulate interactions are occurring. One tentative explanation for the presence of irregular polyhedra may be that flocculation is occuring in the systems whilst the particles are liquid droplets. Droplets in the centre of a floccule may then be sufficiently close to be distorted and this shape could be retained on cooling.

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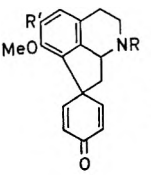
Some pharmacological activities of crotonosine and pronuciferine

SIR,—Crotonosine (Ia) and pronuciferine (Ib) are pro-aporphines isolated from *Croton Linearis* Jacq, a common plant in Jamaica (Haynes & Stuart, 1963; Haynes, Stuart, Barton & Kirby, 1966).



a. R = H, R′ = OH
b. R = Me, R′ = MeO

I

A preliminary estimation of their local anaesthetic activity by the guinea-pig method of Bulbring & Wajda (1945) indicated them to be potent local anaesthetics compared with procaine and lignocaine (Fig. 1). The LD50 of pronuciferine by intraperitoneal injection in mice is 120 mg/kg while that of crotonosine is 1·20 g/kg (Stuart, 1963).

On the guinea-pig isolated ileum, small doses of either compound (pronuciferine, 0·001–1·0 μg/ml; crotonosine, 0·1–10·0 μg/ml) increase the contractions obtained with acetylcholine while larger doses (pronuciferine, 5–200 μg; crotonosine, 100–200 μg/ml) inhibit the actions of acetylcholine and nicotine on the preparation.

With the rat phrenic nerve diaphragm preparation stimulated electrically at a frequency of 6/min (Bulbring, 1946), small doses of either compound (4 μg/ml) increase the response to electrical stimulation while large doses (400 μg/ml) inhibit the response. Pretreatment of the preparation with neostigmine (40 μg/ml) increases the inhibition by crotonosine (400 μg/ml) but reduces the inhibition by pronuciferine (400 μg/ml).

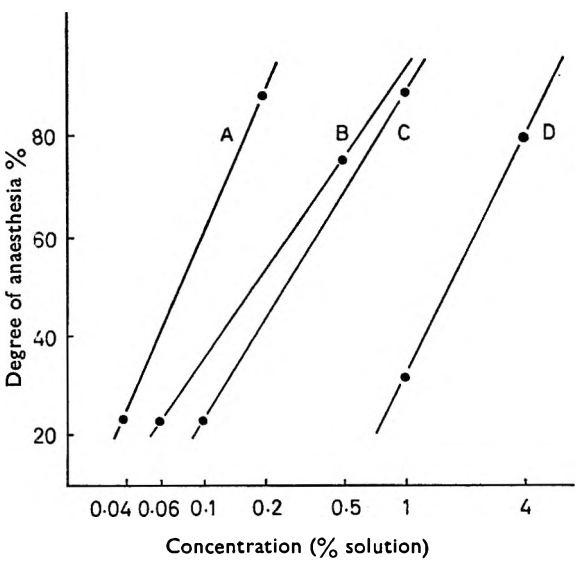


FIG. 1. Mean results of a comparison of local anaesthetic activity of the hydrochlorides of (A) crotonosine, (B) pronuciferine, (C) lignocaine, (D) procaine.

On the toad (*Bufo marinus*) rectus abdominis preparation, both crotonosine and pronuciferine (1–500 μg/ml) increase the contractions obtained with acetylcholine, and at a concentration of 50 μg/ml they antagonise the action of (+)-tubocurarine on this preparation.

Crotonosine causes an initial stimulation followed by depression, but pronuciferine causes only depression on the chick biventer cervicis nerve-muscle preparation (Ginsborg & Warriner, 1960) at 200–400 μg/ml.

The observed inhibitory effects of crotonosine and pronuciferine on these three preparations are consistent with the local anaesthetic activity of the compounds (see de Elio, 1948; Feldberg & Lin, 1949; Kosterlitz & Lees, 1949; Sinha & West, 1953; Green & Hughes, 1966). However, crotonosine and pronuciferine differ from other local anaesthetics in that in small doses they potentiate the chemical and electrical stimulation of these preparations. Whether they first stimulate the release of acetylcholine before inhibiting it is the subject of further investigation.

These two compounds inhibit all the skeletal muscle preparations examined, but seemingly by differing mechanisms. Judging from their difference in behaviour toward neostigmine on the rat phrenic diaphragm preparation and towards the chick biventer cervicis preparation it seems that crotonosine resembles a depolarizing neuromuscular blocking agent while pronuciferine resembles a competitive neuromuscular blocking agent.

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Rubrine C, a pharmacologically active alkaloid from *Phoradendron rubrum*

SIR,—*Phoradendron rubrum* specie *gracile*, is one of many species of the mistletoe family growing wild in Jamaica. The plant is used in local medicine, mainly as a hypotensive. No mention of this specie has been made in the literature, although there are reports of other species of *Phoradendron* (Turnisped, 1851; Deguy, 1901; Delassus & Gaultier, 1907). Having established the presence of alkaloids in *Phoradendron rubrum*, using the colour reactions with Dragendorff and platinic chloride reagents, we now report the isolation and pharmacological examination of one of these alkaloids.

Preliminary investigations indicated that the compound was either a quaternary ammonium, an *N*-oxide, or amphoteric. Attempts to isolate the alkaloid using standard methods failed, as did techniques using adsorption on alumina. Experiments showed the alkaloid to be highly soluble in polar solvents, whether acid or alkaline, indicating the presence of polar group(s). Whilst these could be carboxylic, hydroxyl or phenolic, chemical tests for phenolic alkaloid were negative. Although compounds containing polar group(s) are not eluted easily from Grade I alumina (Heftman, 1961) because of its strong adsorption, they can be eluted with various solvent mixtures from alumina made less adsorptive by hydration. The following method of isolation was therefore devised.

Whole plant was collected, identified, dried and powdered. The powder was exhaustively extracted with ethanol in a percolator, and the ethanol extract stored overnight in a deep freeze. The proteinaceous materials were precipitated and filtered. The resulting extract, which was a thick syrup, was then dissolved in water before adding basic lead acetate and removing excess lead as sulphide. After adding activated charcoal, the aqueous filtrate was reduced to a syrup under reduced pressure. The syrup was dissolved in methanol and non-alkaloidal crystalline materials were removed and the filtrate reduced to a small volume and chromatographed on a Grade III alumina column. An alkaloid termed "Rubrine C" was eluted from the column with a mixture of methanol and chloroform. The alkaloidal base was a semi-solid and extremely hygroscopic. However, the stable crystalline hydrochloride of this base was obtained after a treatment of a cold mixture of methanol in concentrated hydrochloric acid. The hydrochloride was recrystallized from methanol–acetone mixture and dried at 70°.

Rubrine C hydrochloride had m.p. 250° and was optically inactive. Rubrine C found: C, 38·3; H, 7·9; Cl, 22·9; N, 8·95; O, 22·2%: Calculated for ($C_5H_{13}ClNO_2$) C, 38·8; H, 8·4; Cl, 23·0; N, 9·1; O, 20·7%. Chromatography on paper using the descending technique at 28° with butanol–acetic acid–water (2:3:5) 15 hr gave Rf = 0·4; with butanol–acetic acid–water (4:1:5) 15 hr gave Rf = 0·13; and with chloroform–methanol (1:3) 2·5 hr gave Rf = 0·67. The infrared spectrum of Rubrine C hydrochloride in KBr is shown in Fig. 1. In Nujol there were no peaks additional to those seen with KBr. The nmr spectrum of Rubrine C hydrochloride in D_2O at 25° showed two peaks at τ 6·62 (singlet) and τ 5·69 (singlet) respectively.

Rubrine C caused a fall in blood pressure when doses of 0·5–4 mg/kg were injected intravenously into a cat anaesthetized with chloralose. This fall was not blocked by hexamethonium chloride, or by spinalization, but was partially inhibited by atropine at 2 mg/kg body weight. On the Langendorff rabbit heart preparation, the alkaloid at doses from 10–50 μg decreased the force of the heart beat. This effect was removed by washing and was decreased by atropine. Rubrine C had no effect on the rabbit isolated atria. In the

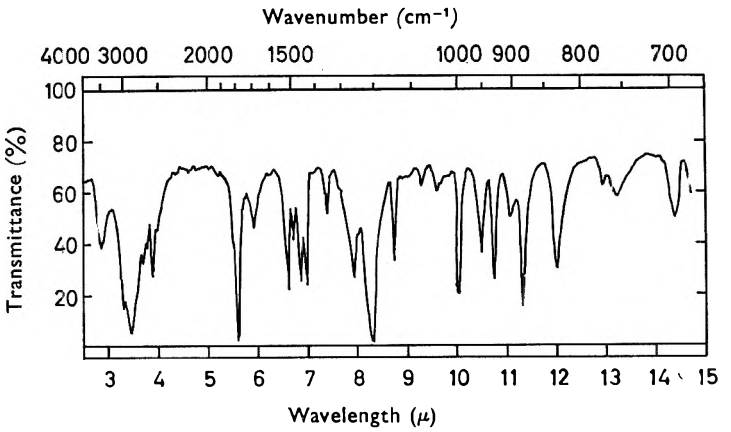

FIG. 1. Infrared spectrum of rubrine C hydrochloride in KBr.

perfused rat hind limb, and rabbit ear, rubrine C caused a prolonged decrease in flow rate at doses of 400 μg. On the guinea-pig ileum, the alkaloid in doses up to 200 μg/ml of bath fluid had no effect. The contractions caused by the spasmogens 5-hydroxytryptamine, nicotine, acetylcholine, barium chloride or histamine were not antagonized or potentiated by that dose of rubrine C.

Rubrine C at 5–40 μg/ml of bath fluid antagonized the contraction of the virgin rat uterus in dioestrus caused by acetylcholine or 5-hydroxytryptamine. Both the pregnant rat uterus and the non-pregnant rat uterus in oestrus, showed sustained contracture of the uterine muscle when the alkaloid, 10 μg/ml of bath fluid, was added. The contracture lasted for about 20 min and prolonged washing was necessary to restore relaxation and spontaneous contractions of the uterus.

This preliminary pharmacology indicates that the activities of this alkaloid are mainly on the cardiovascular system and uterus. From the physical and chemical data this alkaloid might be similar to betaine; however, this is unlikely since betaine is biologically inert, according to Burgen & Hobbiger (1949), and this was confirmed by us on a direct comparison of betaine and rubrine C in various biological tests.

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"Indirect" sympathomimetic effects of L(+)-isomers and desoxy derivatives

SIR,—Molecules which are structurally similar provide a valuable tool for the analysis of drug action. In 1933, Easson & Stedman postulated that for D(−)-isomers of sympathomimetic amines there is a three point interaction with receptors: the phenyl group, the β-hydroxyl group and the amino-group. For L(+)-isomers only a two point interaction is suggested because the β-hydroxyl group is orientated differently and the molecules react essentially as if the β-hydroxyl group were absent. Thus L(+)-isomers are expected to act like desoxy derivatives (Beckett, 1959). This hypothesis has been tested (Patil, La Pidus & Tye, 1967a; Patil, La Pidus, Campbell & Tye, 1967b) and the results indicate that it holds true for indirectly-acting amines in catecholamine-depleted tissue, but not in normal tissues, in which the activity of L(+)-isomers and their corresponding desoxy derivatives appears to be related to differing affinities for the catecholamine uptake sites (Burgen & Iversen, 1965). We now concern ourselves with the effects of various experimental conditions on the relative activities of L(+)-isomers and desoxy derivatives.

The vas deferens of the rat was isolated and suspended in a 10 ml tissue bath containing Tyrode solution at 37·5°, and drug-induced contractions recorded on a smoked drum. Four different procedures were used.

(i) The normal tissue was washed four times, and 15 min after the last wash, drug-induced contractions were recorded.

(ii) The conditions were the same as in (i), but tissue was exposed to 10^{-4} or 3×10^{-4}M noradrenaline, thoroughly washed, and drug-induced contractions recorded. These results were obtained from our previous experiments (Patil & others, 1967a).

(iii) Drug-induced contractions were recorded on reserpine pretreated tissue (5 mg/kg, i.p., 16–24 hr).

(iv) Catecholamine-depleted tissue obtained as above was exposed to 10^{-4}M noradrenaline for 5 min, then washed thoroughly and drug-induced effects recorded. The final bath concentrations of desoxy derivatives and L(+)-isomers were 10^{-4}M and 3×10^{-4}M, respectively. At these concentrations the agents produce maximal effects on this tissue (Patil & others, 1967a). Only

TABLE 1. RESPONSE OF VAS DEFERENS OF RAT TO SYMPATHOMIMETIC AMINES

Drugs	Mean contraction of vas deferens (mm) with s.e. of the mean							
	Normal	N[1]	Normal[2] tissue exposed to noradrenaline	N[1]	Reserpine-pretreated	N[1]	Reserpine-pretreated tissue exposed to noradrenaline	N[1]
α-Methyldopamine, 10^{-4}M (deoxy Cobefrin)	101±8	10	125±9	8	27±5	10	93±10	10
L(+)-Cobefrin, 3×10^{-4}M	35±2	10	52±4	8	14±7	10	61±9	10
Ratio, desoxy/L(+)-isomer	2·8		2·4		1·9		1·51	
P value	<0·001		<0·001		>0·05		<0·05	
m-Tyramine, 10^{-4} M (desoxy norphenylephrine)	116±6	12	136±9	8	17±3	10	99±17	10
L(+)-Phenylephrine, 3×10^{-4}M	73±6	12	86±10	8	28±5	10	68±9	10
Ratio, desoxy/L(+)-isomer	1·5		1·5		0·6		1·4	
P value	<0·001		<0·01		>0·05		>0·05	
Tyramine, 10^{-4}M (desoxy octopamine)	121±11	12	131±5	8	14±5	8	96±16	8
L(+)-Octopamine, 3×10^{-4}M	89±9	12	112±10	8	20±11	9	99±15	9
Ratio, desoxy/L(+)-isomer	1·35		1·16		0·7		0·9	
P value	<0·05		>0·05		>0·05		>0·05	

[1] Number of observations.
[2] Data from Patil & others (1967a).

one drug was tested on any given tissue. Eight to twelve observations were made of each procedure. The significance between the two means was calculated by Student's *t*-test.

In normal, or normal tissue exposed to noradrenaline, the effects of desoxy derivatives were far greater than those of L(+)-isomers. The activity ratio (desoxy derivatives/L(+)-isomer) both in the normal tissue and tissue exposed to noradrenaline remained the same. In the reserpine-pretreated tissue all L(+)-isomers and their desoxy derivatives produced little effect, indicating that these molecules have little or no "intrinsic" activity at sites of direct action, and that their action is possibly due to the release of catecholamine from storage sites. The activity ratios appear to be decreased, possibly due to a large "indirect" component of action in the desoxy derivative compared with that of the L(+)-isomer. In reserpine-pretreated tissue previously exposed to noradrenaline the effectiveness of all compounds was regained by refilling the catecholamine stores. The results are summarized in Table 1.

Kaufman & Friedman (1965) suggested that the action of desoxy derivatives of certain sympathomimetic amines could be attributed to their β-hydroxylated derivatives, which could be easily formed *in vivo* by enzyme dopamine β-hydroxylase. If this were so, it would explain why desoxy derivatives appear more active than L(+)-isomers in normal tissue. However, these possibilities are unlikely because of the following reasons. β-Hydroxylation of desoxy derivatives is a relatively slow process, yet with the present experimental design it took only 30 to 60 sec to obtain the maximal effects of desoxy derivatives. Reserpine abolishes the effects of desoxy derivatives but not of D(−)-isomers (Patil & others, 1967b), which indicates that the action of desoxy derivatives is due to the parent molecule itself rather than to its corresponding D(−)-isomer. In other words, small amounts of D(−)-isomers may be formed but they probably do not contribute significantly to the pharmacological activities of desoxy derivatives in the described conditions.

Our results further substantiate our previous findings that the Easson-Stedman hypothesis (1933) probably holds true at the sites of direct effect, but not when the overall activity of the amines is measured.

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Phenylethyldithiocarbamate: a new dopamine-β-hydroxylase inhibitor

SIR,—It has been shown that disulfiram (tetraethylthiuram disulphide) and its metabolite diethyldithiocarbamate are potent dopamine-β-hydroxylase inhibitors *in vivo* (Goldstein, Anagnoste, Lauber & McKereghan, 1964; Collins, 1965; Carlsson, Fuxe, Hökfelt & Lindqvist, 1966). Dithiocarbamates have been used widely as fungicides and some new derivatives have been investigated in this respect (Thorn & Ludwig, 1962; Rieche, Hilgetag, Martini, Nejedly & Schlegel, 1960). Among the most potent of these was phenylethyldithiocarbamate. The potency of phenylethyldithiocarbamate as a dopamine-β-hydroxylase inhibitor has now been investigated.

Male Sprague-Dawley rats, 200–250 g were used. The weight was within a range of ±10 g in controls and treated animals before an experiment. Phenylethyldithiocarbamate was synthesized according to Rieche & others (1960) and was given subcutaneously as the sodium salt. Noradrenaline in brain and heart was measured (Euler & Lishajko, 1961), and, from the same eluate, brain dopamine was oxidized and measured (Carlsson & Waldeck, 1958). The adrenals from one rat were assayed for adrenaline (Gunne, 1963), the small amount of noradrenaline present being read as adrenaline against the adrenaline standard.

Phenylethyldithiocarbamate was compared with disulfiram for inhibition of the noradrenaline synthesis in whole brain and heart (Table 1). When given

TABLE 1. EFFECT OF REPEATED ADMINISTRATION OF PHENYLETHYLDITHIOCARBAMATE AND DISULFIRAM ON NORADRENALINE AND DOPAMINE CONTENT IN THE WHOLE BRAIN AND HEART.

Disulfiram: 2 × 1 g/kg, orally; doses were given 18, and 3 hr before death. Phenylethyldithiocarbamate: 3 × 200 mg/kg, s.c.; doses were given 51, 27, and 3 hr before death. The results are mean values ± s.e.m., and given as μg/g. Figures in brackets refer to number of experiments.

		Controls (3)	Disulfiram (3)	Controls	Phenylethyldithiocarbamate
Heart	Noradrenaline	0·62 ± 0·02	0·41 ± 0·05*	0·80 ± 0·03 (6)	0·52 ± 0·09*(7)
Brain	Noradrenaline	0·40 ± 0·02	0·22 ± 0·01†	0·40 ± 0·02 (9)	0·21 ± 0·02‡(9)
	Dopamine	0·83 ± 0·06	0·81 ± 0·06	0·67 ± 0·08 (5)	0·74 ± 0·09 (5)

* = $P < 0.05$. † = $P < 0.01$. ‡ = $P < 0.001$.

repeatedly, disulfiram (2 × 1 g/kg, orally) and phenylethyldithiocarbamate (3 × 200 mg/kg, s.c.) caused a decrease of noradrenaline of the same magnitude both in brain and heart, being 45 and 34% respectively. Note that the route of administration and the amount of drug differed in the two experiments. There was no increase of dopamine in the whole brain with either compound. This can be explained by the finding that the dopamine content in the dopamine nerve terminals is unchanged when dopamine-β-hydroxylase is inhibited (Carlsson & others, 1966).

A twofold increase of the dopamine content in the brain stem was found after a single injection (300 mg/kg, s.c.) of phenylethyldithiocarbamate (Table 2). At the same time there was a concomitant decrease of noradrenaline to about 33% of the control value. In this acute experiment no effect was observed on the noradrenaline level in the heart. Phenylethyldithiocarbamate however, produced a marked effect on the catecholamine content in the adrenals, both in

TABLE 2. EFFECT OF PHENYLETHYLDITHIOCARBAMATE ON NORADRENALINE AND DOPAMINE CONTENT IN BRAIN STEM AND HEART

300 mg/kg, s.c. of phenylethyldithiocarbamate was given 3 hr before death. The results are mean values ± s.e.m., and given as μg/g. Figures in brackets refer to number of experiments.

		Controls (5)	Phenylethyldithiocarbamate (5)
Heart	Noradrenaline	0·60 ± 0·05	0·60 ± 0·03
Brain stem	Noradrenaline	0·55 ± 0·02	0·18 ± 0·02‡
	Dopamine	0·34 ± 0·02	0·66 ± 0·08†

† = $P < 0.01$. ‡ = $P < 0.001$.

TABLE 3. ADRENALINE AND DOPAMINE IN THE ADRENALS AFTER SINGLE AND REPEATED ADMINISTRATION OF PHENYLETHYLDITHIOCARBAMATE

Single injection: 300 mg/kg was administered s.c. 3 hr before death. Repeated injections: 200 mg/kg was administrated s.c. 51, 27, and 3 hr before death. The results are mean values ± s.e.m.; noradrenaline is calculated as adrenaline, and the total amount given as μg/2 adrenals. Figures in brackets refer to number of experiments.

	Single injection		Repeated injections	
	Controls (5)	Treated (5)	Controls (6)	Treated (5)
Adrenaline	31·8 ± 0·81	9·5 ± 0·60*	22·7 ± 0·21	8·5 ± 1·80*
Dopamine	0·28 ± 0·03	1·78 ± 0·11*	0·21 ± 0·02	1·11 ± 0·12*

* = $P < 0.001$.

acute (300 mg/kg, s.c.) and chronic (3 × 200 mg/kg, s.c.) experiments (Table 3). The adrenaline content was decreased to about 30 and 40% respectively. Dopamine on the other hand increased five- to sixfold. A similar elevation of dopamine in rat adrenals has been found with diethyldithiocarbamate (Carlsson & others, 1966).

Treatment with disulfiram or with phenylethyldithiocarbamate induced sedation in the rats, but the latter drug seemed to be more potent as judged by the difficulty encountered in arousing the animals. From the present results it appears that phenylethyldithiocarbamate is a dopamine-β-hydroxylase inhibitor. This interpretation is supported by the decrease of noradrenaline and a concomitant increase of dopamine, both in brain stem and adrenals.

The possibility of an *in vivo* decomposition of phenylethyldithiocarbamate should also be pointed out. Such a decomposition is known to occur *in vitro* with sodium diethyldithiocarbamate (Hallaway, 1959). Phenylethylamine may be formed and release noradrenaline from the stores (Jonsson, Grobecker & Holtz, 1966). The phenylethylamine component of this substance may enhance depletion of noradrenaline when dopamine-β-hydroxylase is inhibited.

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Adamantanamines and their derivatives as sensitizing agents for 5-hydroxytryptamine-induced contraction of smooth muscle

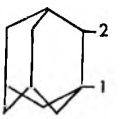
SIR,—It has been shown *in vitro* that smooth muscle contractions induced by 5-hydroxytryptamine (5-HT) or noradrenaline can be sensitized with different drugs. Sigg, Soffer & Gyermek (1963) reported the sensitizing effect of imipramine on the 5-HT- and noradrenaline-induced contraction of the nictitating membrane of the cat. Rossum (unpublished) found that the log concentration-response curve of noradrenaline is shifted to lower concentrations if imipramine or cocaine is used as sensitizer. Offermeier (1965) has described an increase in the response of the rat fundus strip to 5-HT and of the rat vas deferens to noradrenaline after preincubation with imipramine or cocaine. Using cocaine, with some preparations shifts have been obtained with a factor of almost 100. Continuing our study of receptors of neurotransmitters (Wesemann & Zilliken, 1966) we now describe the influence of adamantanamines and their derivatives on 5-HT-induced contractions of the rat isolated fundus strip.

Male rats, strain Wistar II, 160–180 g, starved for 48 hr but given water *ad libitum*, were used for the fundus strip preparation according to Vane (1957). The mucosa was carefully removed to facilitate the washing out of drugs. The muscle strip was incubated in oxygenated Tyrode (10 ml) at 37°. The strip was fixed to one end of a lightly loaded isotonic lever giving about 20 times magnification. Cumulative dose-response curves were obtained by gradually increasing the dose without washing out (Ariëns & de Groot, 1954).

The antiviral compound amantadine (adamantan-1-amine) inhibits the penetration of influenza A_2 virus into the cell (Davies, Grunert, Haff & others, 1964). We were unable to demonstrate a significant inhibition of influenza virus neuraminidase (A_2-Japan virus 1957 E.C.3.2.1.18). However amantadine, in concentrations higher than 10^{-5} M, sensitizes the rat fundus strip to 5-HT (tested with J. Offermeier). With a concentration of 10^{-4} M of the compound the dose-response curve for 5-HT is shifted to the left by a factor of about 10. Maximal sensitization (usually a factor of about 100) is achieved with 10^{-3} M

amantadine. With higher concentrations the ability of the fundus strip to contract in response to 5-HT is lost and cannot be restored by washing out the drugs.

TABLE 1. EFFECT OF ADAMANTANE DERIVATIVES ON THE LOG DOSE-RESPONSE CURVE OF 5-HT OBTAINED ON THE RAT FUNDUS STRIP



Compound	Side-chain	Molarity	Sensitization	α*	No. Strips	No. Curves
Adamantan-1-amine HCl	1-NH_2·HCl	10^{-4} 10^{-3}	10 100	—	58	170
Adamantan-2-amine HCl	2-NH_2·HCl	10^{-4}	150	—	12	62
N-Ethyladamantan-1-amine HBr	1-NH·Et·HBr	10^{-4} 3×10^{-4}	10–15 100	—	15	64
N-(Adamant-1-yl)urea	1-NH·CO·NH_2	10^{-8} 10^{-7} 10^{-6}	10 60–80 500	—	18	54
N-(Adamant-1-yl)-isocyanate	1 — N:C:O	10^{-6}	—	—	13	42
N-(Adamant-1-yl)-isothiocyanate ..	1 — N:C:S	10^{-7}	10	—	9	31
4-(*N*-Adamant-1-yl-carbonyl)morpholine**	1-NH·CO-N⟨O⟩	10^{-8} 10^{-5}	— —	1·25 —	12	54
N-(Adamant-1-yl)-*N'*-cyclohexylurea** ..	1-NH·CO·NH-⟨⟩	10^{-9} 10^{-6} 10^{-5}	— — —	1·15 0·6 0·4	16	64
N-(Adamant-1-yl)-*N'*-phenylurea** ..	1-NH·CO·NH·Ph	10^{-8} 10^{-6} 10^{-5}	— — —	1·1 0·7 0·3	12	51
1,1-Diethylpropylamine		10^{-4}	—	—	9	36

* Relative intrinsic activity. Adamantane compounds were added in Tyrode solution, or where necessary in propylene glycol/Tyrode of ethylene glycol monoethyl ether. Final concentration maximal 0·1% v/v.
** Schlatmann, J. L. M. A. & Schuti, J., to be published.

Table 1 summarizes the sensitizing effect of 12 adamantane compounds tested on the rat fundus strip. 1,1-Diethylpropylamine has been included because of some structural relationship with amantadine. These substances have been synthesized as potential antiviral compounds. Fig. 1 shows a log dose-response curve for 5-HT tested on the rat fundus preparation in the presence of different concentrations of *N*-ethyladamantan-1-amine hydrobromide. Since it is not known how the active adamantane compounds cause sensitization, we prefer to express the shift of the 5-HT log concentration-response curve as a factor rather than as a change in affinity $1/K_A$, the value K_A being the dissociation

constant of the drug-receptor complex. The log dose-response curve obtained resembles the type for a competitive synergism more than the theoretical dose-response curve for an agonist A combined with various concentrations of a sensitizing compound B (Ariëns, Simonis, & Rossum, 1964). In the latter case, a parallel shift to the left would be expected when increasing concentrations of B (adamantane compounds) are used, until a limit is reached.

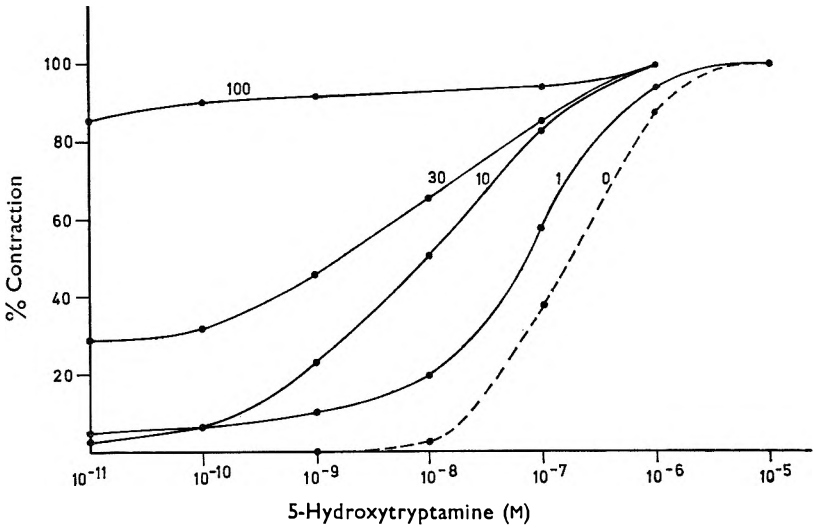


FIG. 1. Cumulative log dose-response curves of 5-HT in the presence of various concentrations ($\times 10^{-5}$M) of *N*-ethyladamantan-1-amine HBr tested on the rat fundus preparation.

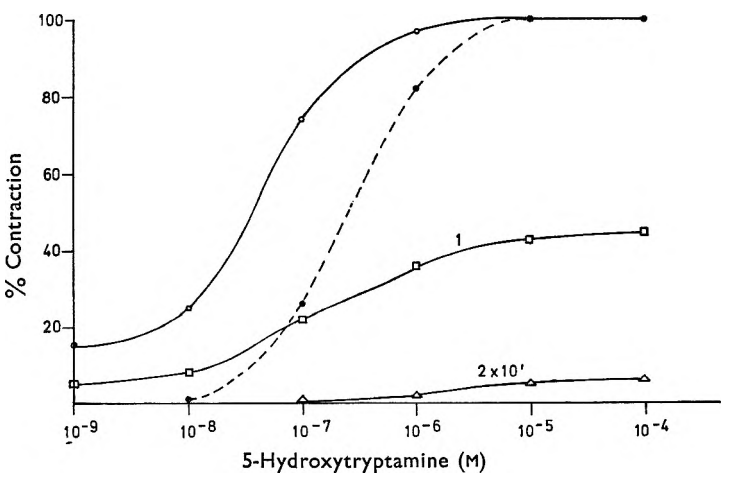


FIG. 2. Cumulative log dose-response curves of 5-HT in the presence of 10^{-4}M adamantan-1-amine after various incubation times with 3×10^{-4} dibenamine, tested on the rat fundus strip preparation. 5-HT log dose-response curve ●-----●; 5-HT log dose-response curve sensitized by 10^{-4}M adamantan-1-amine after incubation with 3×10^{-4} dibenamine for 0 min ○——○, 5-HT log dose-response curve sensitized by adamantan-1-amine after incubation with 3×10^{-4}M dibenamine; incubation time 10 min □——□, incubation time 20 min △——△.

Some of the compounds tested, though inactive as sensitizers, influence the relative intrinsic activity α. With *N*-(adamant-1-yl)-*N'*-cyclohexylurea the maximum height of the 5-HT dose-response curve is increased at low concentrations of the adamantane compound ($\alpha = 1{\cdot}15$). A decrease of the maximum height to 40% of the height of the curve for 5-HT alone occurs at a concentration of 10^{-5} M. Cocaine and imipramine produce sensitization to 5-HT in concentrations between 10^{-9} and 3×10^{-7} M and 10^{-8} and 10^{-6} M respectively (Offermeier, 1965). Log concentration-response curves for 5-HT on preparations already sensitized by adamantane compounds can be shifted further towards lower concentrations by incubating the fundus strip with 10^{-8} and 3×10^{-8} M cocaine (Table 2) whilst with imipramine no significant further increase or decrease could be obtained.

TABLE 2. EFFECT OF COCAINE ON THE LOG DOSE-RESPONSE CURVE OF 5-HT ON THE RAT FUNDUS STRIP* SENSITIZED BY ADAMANTANE COMPOUNDS

Compound	Concentration M	Concentration cocaine M	Sensitization factor†
Adamantan-1-amine HCl	10^{-5}	3×10^{-8}	10
N-Ethyladamantan-1-amine HBr	10^{-4}	10^{-6}	10
N-(Adamant-1-yl)urea	10^{-7}	10^{-8}	10

* Each experiment used 6 strips.

† Additional sensitization by cocaine of the 5-HT response already sensitized the adamantane compounds. Substances were added in Tyrode solution, only *N*-(adamant-1-yl)urea was dissolved in ethylene glycol monoethyl ether, final concentration maximal 0.1% v/v.

According to Woolley (1958) the 5-HT-receptor interaction opens a "valve" for the Ca-ion transport, which leads to the contraction of muscle. Contractions produced by just adding Ca^{2+} or Mg^{2+} ions in the rather high concentrations from 10^{-3} to 10^{-1} M to the organ bath were not influenced by dibenamine hydrochloride which irreversibly blocks 5-HT receptors. Since Ca-induced contractions are not influenced by dibenamine, this blocking agent may act directly on the 5-HT receptor and not interfere with the stimulus-event chain, that is to say, the Ca-ion transport, the polarization, depolarization, and the effector system. Inasmuch as the 5-HT concentration-response curves of the tissue sensitized by adamantanamine were found to decline after various incubation times with dibenamine (Fig. 2), adamantanamine apparently acts at the receptor level and not at the stimulus-effect chain. Perhaps the adamantane compounds react with storage sites of 5-HT or influence the drug-receptor metabolism thus increasing the concentration of 5-HT in the vicinity of the receptor.

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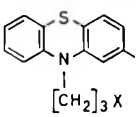
Activity correlations and the mode of action of aminoalkylphenothiazine tranquillizers

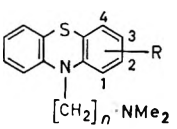
SIR,—Potent tranquillizing activity in the aminoalkylphenothiazine series is generally limited to those compounds (I) which have a substituent in the 2-position of the phenothiazine nucleus, and in which the phenothiazine nitrogen



(I) R = H, Cl, CF_3 etc.
X = Tertiary amino-group

atom and the terminal amino-group are separated by a trimethylene chain (Gordon, Craig & Zirkle, 1964). The amino-group and part of the side-chain may be incorporated into a ring system such as piperidine. Within this restricted range of structural variation, numerous correlations have been reported between

TABLE 1. CATALEPTIC ACTION, SURFACE ACTIVITY, IONIZATION CONSTANT, WATER SOLUBILITY AND EFFECT ON ATPase OF DIMETHYLAMINOALKYLPHENOTHIAZINES



R	*n*	ED50 (mg/kg) for catalepsy	Surface-active concn (μM)	I50 (μM) for ATPase inhibition	pK_a	Solubility (μM) of free base in water
H	3	25	800	250	9·4	50
1—Cl	3	none at 50	500	150	9·4	12
2—Cl	3	4	240	80	9·3	8
3—Cl	3	none at 20	180	120	9·2	10
4—Cl	3	none at 50	300	50	9·2	11
2—CF_3	3	2·5	70	100	9·2	5
4—CF_3	3	none at 50	50	80	9·3	7
2—Cl	2	none at 20	200	150	8·6	15
2—Cl	4	none at 25	140	200	9·7	5

ED50 is the dose of drug required to cause catalepsy in 3 out of 6 mice 1 hr after intravenous injection (Taeschler & Cerletti, 1958).

The surface-active concentration is that required to lower the surface tension of 10 mM sodium phosphate (pH 6·97) by 5 dynes/cm when measured with a Du Noüy tensiometer and platinum ring.

A rat brain microsomal suspension treated with sodium deoxycholate was used as a source of $(Na^+ + K^+)$-activated ATPase (Järnfelt, 1964). I50 is the concentration of compound required to inhibit the ouabain-sensitive fraction of the activity by 50% when measured at 37° in 30 mM tris buffer (pH 7·5) in the presence of 20 mM KCl, 100 mM NaCl, 5 mM $MgCl_2$ and 2·5 mM ATP.

The pK_B was derived from the pH dependence of the water solubility (Green, 1967).

the tranquillizing potency and the activity in isolated chemical or biochemical systems. Some, such as the correlations of tranquillizing potency and surface activity (Seeman & Bialy, 1963), or inhibition of $(Na^+ + K^+)$-activated ATPase (Davis & Brody, 1966), have been cited as evidence that tranquillization is causally connected with these simpler actions.

The results in Table 1 show that when the nuclear substituent is transferred to some other position in the ring, or when the polymethylene side-chain is altered in length, the marked fall in tranquillizing activity, assessed here from the ED50 for catalepsy, is not accompanied by any corresponding decrease in either surface activity or inhibitory potency against $(Na^+ + K^+)$-activated ATPase. Nor are there any marked changes in ionization constant or water solubility (cf. Green, 1967). The failure of any of the above systems to discriminate between the potent tranquillizers and the closely related but much less active compounds casts doubt on the significance of the activity correlations mentioned earlier.

The possibility that compounds with substituents in other than the 2-position fail to act as tranquillizers because they do not enter the brain in sufficient concentration has been largely excluded by the finding that all four chloro-10-dimethylaminopropylphenothiazines accumulate to roughly the same extent (13, 20, 34 and 26 μmole/kg for the 1-, 2-, 3- and 4-chloro isomers respectively) 1 hr after subcutaneous injection of equal doses (20 μmole/kg) into mice. The compounds were extracted with heptane from a basified brain homogenate (Wechsler & Forrest, 1959) and estimated spectrophotometrically (Salzman & Brodie, 1956). It is difficult to be certain that the compounds extracted are the unchanged drugs and not inactive metabolites, but the ultraviolet spectrum of each extract was the same as that of the pure drug. This eliminates the possibility of oxidation to sulphoxide or sulphone; but it remains to be established that there is no selective alteration of the side-chain, that might not change the spectra but could profoundly modify the biological activity.

Until a satisfactory explanation can be offered why a 2-substituent and a trimethylene side-chain are essential for potent tranquillizing activity in the aminoalkylphenothiazine series, activity correlations should be viewed with some scepticism as evidence for particular mechanisms of tranquillization.

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Letters to the Editor

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