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The effect of solvents on the potency of chlordiazepoxide, diazepam, medazepam and nitrazepam

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ED50 values for loss of righting reflex in mice have been determined for a series of benzodiazepines after intraperitoneal injection of the drugs in various vehicles. The vehicles used greatly modified the ED50 values obtained. The effects obtained were due either to a failure of the vehicle to achieve or maintain complete solubilization of the drug, or to the pharmacological action of the vehicle modifying that of the drug. Diazepam, medazepam and nitrazepam are insoluble in water, but are soluble in dimethylsulphoxide (DMSO) and in aqueous vehicles containing glycofurol or propylene glycol. Chlordiazepoxide hydrochloride is water-soluble. Similar ED50 values were obtained in experiments where the benzodiazepines were injected in an aqueous carboxymethylcellulose suspension and in experiments where the drugs were dissolved in DMSO. Lower ED50 values were obtained when the drugs were dissolved in vehicles containing propylene glycol or glycofurol. The increase in potency of the benzodiazepines could be ascribed either to the solubilization of the drugs or to the synergistic pharmacological activity of the solvents. An ED50 value for diazepam, which was not influenced by the pharmacological activity of the solvent, could be obtained using the vehicles containing either glycofurol or propylene glycol. For medazepam and nitrazepam, the solvent mixtures containing propylene glycol and glycofurol respectively were required to avoid drug-solvent interactions.

In clinical practice the benzodiazepines are administered parenterally for the therapy of epileptiform seizures (Gastaut, Naquet & others, 1965), muscle spasm and tetanus (Weinberg, 1964) and for premedication and the induction of anaesthesia (see Knight & Burgess, 1968, for references).

Chlordiazepoxide is manufactured as the hydrochloride salt which is freely soluble but unstable in aqueous solution (Randall, 1961). As the benzodiazepines, diazepam, nitrazepam and medazepam, are insoluble in water, other solvents are used in formulations of these drugs for injection. Formulations containing glycofurol (Spiegelberg, Schläpfer & others, 1956) and propylene glycol have been used commercially as vehicles for diazepam.

Preliminary screening of glycofurol and propylene glycol has shown that, like the benzodiazepines, they may produce hypnosis, motor inco-ordination, depression of polysynaptic reflexes and ataxia (unpublished observations). In the present experiments the interactions of various solvents with the benzodiazepines, chlordiazepoxide HCl, diazepam, medazepam (Ro5-4556) and nitrazepam have been examined using loss of the righting reflex in mice as a measure of pharmacological activity.

METHODS

The benzodiazepines, chlordiazepoxide HCl (Librium), diazepam (Valium), medazepam (Nobrium) and nitrazepam (Mogadon), were formulated in one of the following vehicles for intraperitoneal injection:

(a) Dimethylsulphoxide. (b) Propylene glycol (45% v/v), ethanol (10% v/v), benzyl alcohol (1·5% v/v), sodium benzoate (9·8% w/v), benzoic acid (0·24% w/v) in water. (c) Glycofurol (45% v/v), ethanol (10% v/v), benzyl alcohol (1·5% v/v) in water. (d) A suspending vehicle of sodium carboxymethylcellulose (0·5% w/v), Polysorbate 80 (0·5% v/v), phenylmercuric nitrate (0·001% v/v) in water. In the text these formulations are referred to as DMSO, propylene glycol solvent, glycofurol solvent and the methylcellulose vehicle respectively. Vehicles (b) and (c) have been used as solvents for diazepam in a commercial preparation for parenteral use (Valium ampoules, Roche).

Each of the benzodiazepines is soluble in formulations (a), (b) and (c) at a strength of 5 mg/ml of vehicle. Solutions using these vehicles were injected intraperitoneally, without dilution, using a micro-syringe. Drugs in formulation (d) were injected after suitable dilution using a 1·0 ml tuberculin syringe.

White Swiss mice (Commonwealth Serum Laboratories), of either sex, 20–30 g, were weighed individually and the various formulations administered intraperitoneally. Animals were tested for loss of righting reflex for 3 min during a test period of 13 min from the time of injection. ED50 values were calculated by the method of Litchfield & Wilcoxon (1949), using groups of 10 or 20 mice at each dose. Drug formulations were prepared immediately before each experiment.

RESULTS

Table 1 shows the ED50 values for chlordiazepoxide HCl, diazepam, nitrazepam and medazepam (at 5 mg/ml) in the different vehicles. The relative potencies of the benzodiazepines are also shown (chlordiazepoxide HCl = 1). The ED50 values and the relative potencies of the benzodiazepines are similar in the methylcellulose vehicle and in DMSO but lower values were obtained with the other vehicles.

Table 1. *ED50 values (mg/kg) for benzodiazepines (5 mg/ml) in various formulations.*

	Methylcellulose vehicle		DMSO		Propylene glycol solvent		Glycofurol solvent	
	ED50	RP	ED50	RP	ED50	RP	ED50	RP
Chlordiazepoxide HCl	35·4 (37·2–33·5)	1·0	34·8 (40·0–30·3)	1·0	30·8 (37·0–25·7)	1·0	14·1 (17·6–11·3)	1·0
Diazepam	25·6 (33·2–19·7)	1·4	18·5 (23·5–14·8)	1·9	2·8 (3·3–2·0)	11·0	2·9 (3·5–2·4)	4·8
Medazepam	42·2 (51·3–33·1)	0·8	31·5 (34·6–28·6)	1·1	16·5 (17·8–15·3)	1·9	10·5 (12·1–9·1)	1·3
Nitrazepam	30·2 (36·2–25·1)	1·2	26·0 (32·5–20·8)	1·3	23·0*	1·3	7·8 (9·1–6·6)	1·8

ED50 values with 95% confidence limits for loss of righting reflex in mice produced by the benzodiazepines in various vehicles. The formulations were injected intraperitoneally, each benzodiazepine being used in a concentration of 5 mg/ml of vehicle. Relative potencies (RP) (chlordiazepoxide = 1) are shown. The ED50 value for nitrazepam (*) is approximate as the probit line was non-linear (see text).

With each of the benzodiazepines, the rank order of the ED50 values varies with the vehicle used. The mean values decrease in the order, methylcellulose vehicle, DMSO, propylene glycol solvent and glycofurol solvent. The exception is diazepam, where similar ED50 values were obtained with propylene glycol solvent and glyco-

furol solvent, though both were markedly different from the values for diazepam in the other solvents.

The variation in the results obtained suggests that the potency of the benzodiazepines is influenced by the vehicle used. In experiments where the effects of the vehicles alone were tested, with the exception of the methylcellulose vehicle, each solvent produced a loss of righting reflex in mice. Table 2 shows ED50 values and slope functions for the solvents, expressed as ml/kg. Table 2 also shows the ED50 values for the drug-vehicle combinations, expressed as ml/kg, when the benzodiazepines were used at 5 mg/ml in the various vehicles. In this comparison it can be

Table 2. *ED50 values (ml/kg) for the vehicles and for the benzodiazepine (5 mg/ml) formulations.*

	Methylcellulose vehicle		DMSO		Propylene glycol solvent		Glycofurol solvent	
	ED50	Slope function	ED50	Slope function	ED50	Slope function	ED50	Slope function
Solvent alone	Greater than 30 ml/kg		14·8 (18·5–11·9)	1·9 (2·1–1·7)	9·6 (11·0–8·4)	1·6 (2·0–1·3)	3·3 (3·6–3·0)	1·3 (1·6–1·1)
Chlordiazepoxide HCl (5 mg/ml)	7·1 (7·4–6·7)	1·2 (1·3–1·1)	7·0 (8·0–6·1)	1·7 (2·0–1·4)	6·1 (8·4–5·2)	1·4 (1·7–1·2)	2·8 (3·5–2·3)	1·3 (1·4–1·2)
Diazepam (5 mg/ml)	5·1 (6·6–3·9)	2·1 (2·6–1·7)	3·7 (4·7–2·9)	1·9 (2·5–1·5)	0·57 (0·66–0·40)	1·6 (2·0–1·2)	0·59 (0·70–0·48)	1·5 (1·8–1·3)
Medazepam (5 mg/ml)	8·4 (10·5–6·6)	2·2 (2·2–1·8)	6·3 (6·9–5·7)	1·3 (1·6–1·1)	3·3 (3·6–3·0)	1·2 (1·3–1·2)	2·1 (2·4–1·8)	1·4 (1·5–1·1)
Nitrazepam (5 mg/ml)	6·6 (7·2–5·0)	1·9 (2·3–1·7)	5·2 (6·5–4·2)	1·8 (2·6–1·3)	4·6*	—	1·6 (1·8–1·3)	1·6 (2·0–1·2)

As in Table 1: ED50 values, slope functions and 95% confidence limits for the vehicles alone and for the benzodiazepine formulations expressed as ml/kg. The ED50 for nitrazepam in propylene glycol solvent (*) is approximate owing to non-linearity of the probit plot.

seen that the ED50 value for DMSO (14·8 ml/kg) is much higher than that obtained with each benzodiazepine-DMSO combination. With the propylene glycol solvent the ED50 value is 9·6 ml/kg, and this again is in excess of the values obtained with each of the drug-solvent combinations. The ED50 value for the glycofurol solvent (3·3 ml/kg) is similar to the values obtained when chlordiazepoxide HCl and medazepam were dissolved in this vehicle (2·8 and 2·1 ml/kg respectively). This suggests the possibility of an interaction between these drugs and the glycofurol solvent.

To evaluate the way in which these vehicles might influence the potency of the drugs, both the pharmacological and the physicochemical properties of the vehicle and the drug-vehicle combination must be considered. Gaddum (1953) described a method for evaluating the interaction between two drugs that have a similar pharmacological action. Using cartesian coordinates the ED50 value for one drug is plotted on one axis and the ED50 value for the second drug is plotted on the other axis (Points A & B in Fig. 1a). ED50 values for combinations of the two drugs are plotted in the field. Points above the horizontal line AC represent antagonism of drug A by drug B; points to the right of the vertical line BC represent antagonism of drug B by drug A, and points within the rectangle OACB represent synergism between the two drugs. If a point falls on either line AC or BC, no interaction is present at that ratio.

A plot evaluating the interaction between the water-soluble drug chlordiazepoxide HCl and the glycofurol solvent is shown in Fig. 1b. The broken line in this Figure and also in Fig. 1c and 1d represents a constant ratio of 5 mg of the benzodiazepine to 1 ml of the solvent, as used initially (Table 1). The ED50 for chlordiazepoxide HCl in water is 35·4 mg/kg, while that for glycofurol solvent alone is 3·3 ml/kg. The

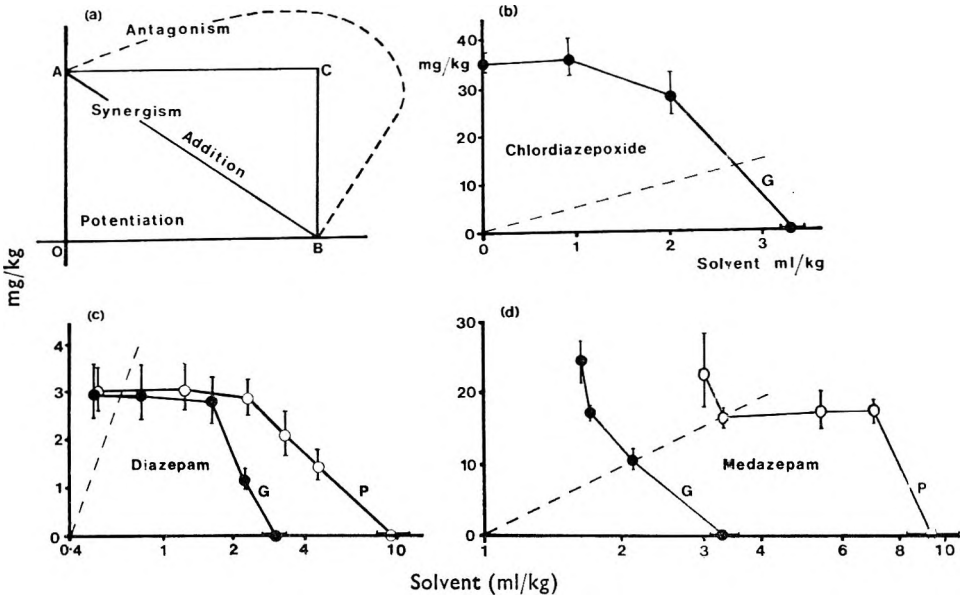

FIG. 1. (a) Diagrammatic representation (redrawn from Gaddum, 1953) of a method for evaluating interactions between two drugs (see text). ED50 values with 95% confidence limits for loss of righting reflex in mice produced by combinations of (b) chlordiazepoxide HCl, (c) diazepam and (d) medazepam with glycofurol solvent, G (●) and propylene glycol solvent, P(○). The broken lines in (b) (c) and (d) represent a constant ratio of drug to solvent of 5 mg/ml.

ED50 value for chlordiazepoxide HCl when administered with a constant dose of either 1 or 2 ml/kg of glycofurol solvent is shown. At 1 ml/kg of the glycofurol solvent, no interaction is apparent, while at 2 ml/kg synergism occurs, as indicated by a departure from horizontal. At a ratio of 5 mg/ml (broken line) marked synergism is apparent and the ED50 value quoted in Table 1 for the chlordiazepoxide HCl-glycofurol solvent combination is the result of synergistic action.

When similar plots are produced with diazepam (Fig. 1c), which is insoluble in water, it is not possible to obtain a point on the vertical axis. However ED50 values for diazepam with constant doses of 0·5, 1·0 and 2·0 ml/kg of both glycofurol solvent and propylene glycol solvent, lie on a horizontal line. This indicates that there is a range of drug-solvent ratios where interaction is absent. Extrapolation of the horizontal line may therefore give an estimated ED50 value of 2·9 mg/kg for diazepam. The broken line (ratio 5 mg/ml) passes through the horizontal component of both lines, indicating an absence of interaction with either propylene glycol solvent or glycofurol solvent at this drug-solvent ratio.

With medazepam, through the full range of solubility in the glycofurol solvent, no horizontal component is apparent (Fig. 1d) but with the propylene glycol solvent there is a horizontal component and extrapolation from this line gives an estimated ED50 of 16·5 mg/kg. It should be noted that the 5 mg/ml line passes through the horizontal component of this line indicating an absence of interaction with the propylene glycol solvent at this drug-solvent ratio. The upper point on each line in Fig. 1d represents a point where, using a fixed dose of solvent, difficulty was experienced in maintaining solubilization of the drugs. The result is an upward distortion of the line and a widening of the 95% confidence limits. This effect is possibly due

to precipitation of the drug at the site of administration and a resulting decrease in potency.

Difficulties were experienced when attempting to assess the ED50 values of nitrazepam in the presence of the propylene glycol solvent. Fig. 2 shows the probit lines obtained for nitrazepam with different doses of the propylene glycol solvent. As each line is non-linear, an ED50 value could not be calculated. However, the probit line obtained for nitrazepam in the methylcellulose vehicle (broken line, Fig. 2) lies close to the second upward deflection of each of the probit lines obtained in the presence of the propylene glycol solvent.

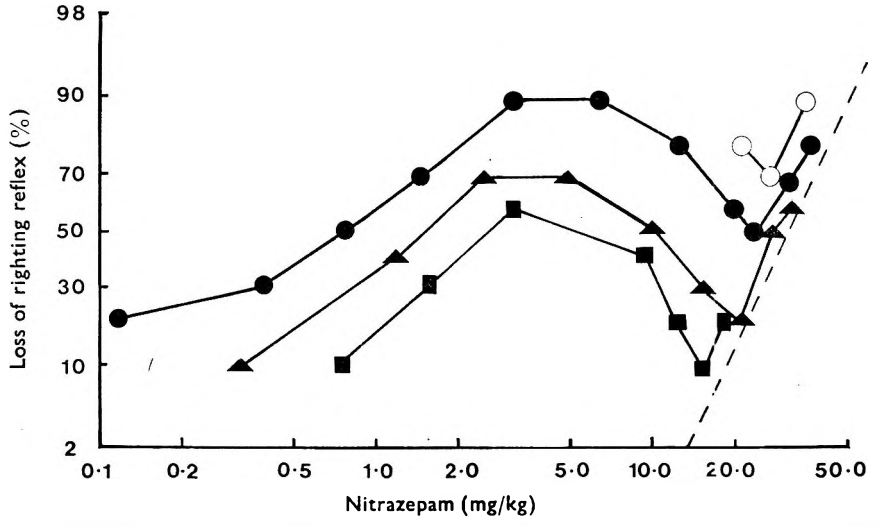

FIG. 2. Probit analysis showing the loss of righting reflex in groups of 10 mice produced by varying doses of nitrazepam in the presence of constant doses of the propylene glycol solvent (7 ml/kg, ○; 6 ml/kg, ●; 5 ml/kg, ▲; and 3 mg/kg, ■). The broken line to the right shows the position of the line of best fit of the probit plot for nitrazepam suspended in the methylcellulose vehicle.

As it was not possible to calculate the ED50 values for nitrazepam in the propylene glycol solvent, further experiments were made. It was found that if the dose of nitrazepam was kept constant for a given test and the amount of propylene glycol solvent was varied, ED50 values could be obtained for the solvent in the presence of the drug. The use of a constant dose of nitrazepam with a variable dose of the propylene glycol solvent does not alter the basic method of studying an interaction, but avoids extending the doses of nitrazepam into a range where multiple actions are observed. In Fig. 3 the interaction between nitrazepam and the propylene glycol solvent is shown. The vertical axis represents the various fixed doses of nitrazepam administered when various ED50 values for the propylene glycol solvent were obtained. The horizontal axis represents the actual concentration of nitrazepam in the propylene glycol solvent at each point. This method of plotting demonstrates the variation in potency of nitrazepam with varying strengths of solution, no horizontal component being present in the plot.

When ED50 values were obtained with constant doses of DMSO in combination with varying doses of nitrazepam, a plot of ED50 values against strength of solution was obtained (Fig. 3). It is apparent that DMSO, like propylene glycol solvent, is unsatisfactory as a solvent as there is no horizontal component to the line.

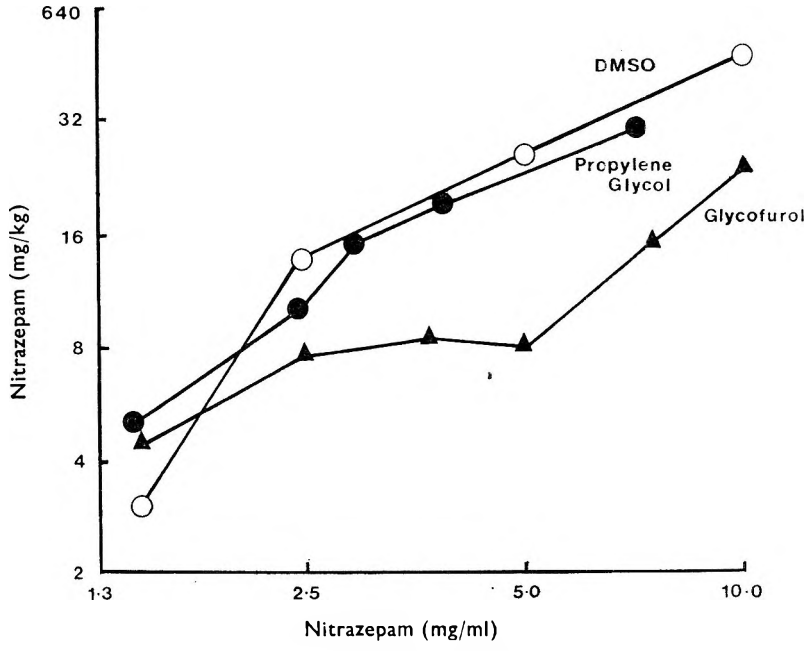

FIG. 3. Dose of nitrazepam required to produce loss of righting reflex in 50% of mice (ordinate) plotted against the concentration of this drug in various solvents (abscissa). In the case of DMSO (○) and the glycofurol solvent (▲), the values plotted on the ordinate are ED50 values for nitrazepam. In the case of the propylene glycol solvent (●) the points on the ordinate represent constant doses of nitrazepam administered in combination with varying doses of propylene glycol solvent.

When ED50 values were obtained for nitrazepam in the presence of the glycofurol solvent and plotted in the same way as for the other two solvents, a horizontal component was present (Fig. 3). Extrapolation of the horizontal component of this line gives an ED50 value for nitrazepam of 7·7 mg/kg.

DISCUSSION

Of the benzodiazepines considered, chlordiazepoxide HCl alone is water-soluble. In most animal tests, these drugs have been given by mouth. The insoluble drugs have usually been administered in some form of suspension (see Zbinden & Randall, 1967, for review). To produce the pharmacological effects under test, a drug must achieve a critical concentration in the biophase which surrounds the active sites involved. For the drug to pass from the site of administration to the site of action a number of criteria must be satisfied. The solvent must maintain solution of the drug *in vitro*; it must ensure continued solution of the drug at the site of administration; it must not impede the uptake of the drug into the circulation, and finally, it must not interfere with the drug at its site of action.

In the situation studied, with chlordiazepoxide HCl, all criteria are satisfied when the drug is given in water. If chlordiazepoxide is given with the solvent DMSO or other pharmacologically active vehicles, its action may be potentiated.

With diazepam, medazepam and nitrazepam, which are all insoluble in water, attention must be paid to the choice of vehicle. The use of an aqueous vehicle, containing suspending agents, results in consistently high ED50 values when the

responses are compared with those obtained with other solvents. This suggests that uptake into the circulation is slow. A probable explanation is slow dispersion of the drug from undissolved particles at the site of injection. The relative potencies obtained when using the methyl cellulose vehicle (Table 1) are similar to those given by Zbinden & Randall (1967) for oral administration of the drugs, where a similar pharmacological test was used (chlordiazepoxide HCl = 1·0; diazepam = 1·6; medazepam = 0·44 and nitrazepam = 0·84). The doses required were approximately ten times greater by mouth than parenterally. This similarity in relative potency probably indicates that absorbtion of the drugs from the lumen of the gastrointestinal tract and from the peritoneal surfaces involves a similar mechanism. The difference in the absolute potency is possibly the result of different overall rates of absorption.

When the solvent DMSO is used, similar ED50 values are obtained to those found with the methylcellulose vehicle. Precipitation of the water-insoluble drugs at the injection site is a probable explanation of these results, although inspection of the peritoneal cavity of the animals did not reveal any gross precipitation. DMSO, through hydrogen bonding, has an extremely high affinity for water (MacGregor, 1967). Contact with the body fluids leads to disruption of the anhydrous polymerization of DMSO and a rapid dispersion of this substance in the body (Rammler & Zaffaroni, 1967). This disruption of the anhydrous structure destroys the ability of DMSO to maintain solution of a non-ionized solute (Parker, 1965). Such an effect would result in precipitation of the benzodiazepines at the injection site. Thus DMSO is a totally inadequate solvent for the maintenance of solution of these drugs in contact with body fluids. Furthermore, DMSO has a variety of pharmacological actions that could interfere with the actions of the drugs (Leake, Rosenbaum & Jacob, 1967).

The propylene glycol solvent has relatively low biological activity and is therefore potentially suitable as a solvent for benzodiazepines that are insoluble in water. It is suitable for diazepam, and also for medazepam which has relatively low biological activity. The absence of constant ED50 values with different strengths of nitrazepam in the propylene glycol solvent, makes this particular drug-solvent combination of limited use.

The reversal of the depressant action of nitrazepam in the presence of the propylene glycol solvent is of considerable interest. Sternbach, Randall & Gustafson (1964) have described an excitatory action of nitrazepam when large doses are given by mouth. Finney (1947) has described a dose response curve similar to that obtained in the present experiments when the antifungal action of tetramethylthiuram was tested. Finney suggested that the non-linear response could be due to competition between a dissociated and an un-dissociated form of this compound, the result being a fall in potency in certain concentration ranges. Such an explanation could fit the effects observed with nitrazepam. However, the phenomenon might also be explained in terms of a differential depression of central excitatory and inhibitory neurons in the central nervous system. In the context of this paper, it appears that the effects observed are properties of the drug nitrazepam, and the solvent is merely modifying these.

The glycofurol solvent dissolves each of the benzodiazepines, but has the highest biological activity of the vehicles tested. It potentiates the action of medazepam

when the drug is given at the limit of solubility. This interaction makes the combination unsuitable if solvent effects are to be excluded.

Both diazepam and nitrazepam are more potent than medazepam and solutions may be prepared in which the potency of the drugs is not influenced by the presence of the solvent. Due to interactions, other solvents are unsatisfactory and the glycofurol solvent appears to be the vehicle of choice for nitrazepam in the situation studied.

Table 3. *ED50 values for benzodiazepines.*

	ED50	Slope function	Relative potency	Solvent*
Chlordiazepoxide HCl ..	35·4 (37·2–33·5)	2·1 (2·9–1·5)	1·0	Water
Diazepam	2·9 (3·5–2·4)	1·6 (1·8–1·3)	12·2	Glycofurol solvent or propylene glycol solvent
Medazepam	16·5 (17·8–15·3)	1·2 (1·3–1·1)	2·1	Propylene glycol solvent
Nitrazepam	7·7 (9·1–6·6)	1·6 (2·0–1·3)	4·6	Glycofurol solvent

ED50 values (mg/kg), slope functions, 95% confidence limits and relative potencies (chlordiazepoxide HCl = 1) are shown for chlordiazepoxide HCl, diazepam, medazepam and nitrazepam. To avoid interactions at solution strengths of 5 mg/ml.

Table 3 shows the ED50 values, with slope functions and 95% confidence limits, for each drug after solvent effects have been excluded. The vehicles needed to avoid drug-solvent interactions are shown in each case. Relative potencies are also shown (chlordiazepoxide HCl = 1).

These experiments show that the absolute and relative potencies of the benzodiazepines chlordiazepoxide HCl, diazepam, medazepam and nitrazepam, when administered parenterally, may be modified by the solvents used. These interactions can cause either a reduction or an enhancement of the actual potencies of the compounds. Glycofurol solvent, propylene glycol solvent, methylcellulose vehicle and DMSO have been used as vehicles for these drugs. The present experiments indicate that their biological activity and their limitations as solvents must be considered whenever they are used. A simple means of identifying solvent effects is to vary the amount of solvent used and to test for a change in the potency of the drug. If a change in potency is apparent, further testing is necessary to identify the nature of the effect.

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Effect of nigral lesion on chlorpromazine-induced acceleration of dopamine synthesis from [^{14}C]tyrosine*

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The nigro-neostriatal dopamine pathway of the rat brain was subjected to a unilateral stereotaxic lesion at the level of the hypothalamic-mesencephalic junction. Fifteen days after the operation endogenous dopamine and[^{14}C]dopamine formed *in vivo* from [^{14}C]tyrosine were reduced to about 15% in the striatum ipsilateral to the lesion. Twenty-four h after the lesion the contents of endogenous and labelled dopamine were about the same in the striata of both sides. Chlorpromazine (15 mg/kg) accelerated several fold the accumulation of [^{14}C]dopamine formed from [^{14}C]tyrosine in the striatum on the intact side. However, in the striatum on the side of the lesion, chlorpromazine did not increase the accumulation of [^{14}C]dopamine. The results indicate that chlorpromazine accelerates dopamine synthesis in the striatum by an indirect mechanism, presumably by activating the nerve impulse flow in the nigro-neostriatal dopamine pathway.

Histochemical and biochemical studies have demonstrated a dopamine neuron system extending from the substantia nigra in the brain stem to the nucleus caudatus and putamen (Andén, Carlsson & others, 1964; Dahlström & Fuxe, 1964; Bertler, Falck & others, 1964). Clinical and experimental findings indicate that the nigro-neostriatal dopamine pathway is involved in extrapyramidal motor control (Ehringer & Hornykiewicz, 1960; Carlsson, 1964; Poirier & Sourkes, 1965). Various independent groups have demonstrated that dopamine synthesis in the striatum is accelerated by chlorpromazine (Andén, Roos & Werdinius, 1964; Bernheimer & Hornykiewicz, 1965; Nybäck & Sedvall, 1969), an effect that has been ascribed to a feedback activation of the dopamine neurons induced by a blockade of dopamine receptors (Carlsson & Lindqvist, 1963).

The present investigation was undertaken to test the hypothesis that the chlorpromazine-induced acceleration of dopamine synthesis involves an activation of the nigro-neostriatal dopamine pathway. Nerve impulse activity in the left nigro-neostriatal dopamine pathway was interrupted by a stereotaxic lesion. The accumulation of [^{14}C]dopamine in the left and right striatum during an intravenous infusion of [^{14}C]tyrosine was taken as an index of endogenous dopamine synthesis (Sedvall, Weise & Kopin, 1968; Nybäck & Sedvall, 1970).

METHODS

Male Sprague-Dawley rats, 160–180 g, were used. Under pentobarbitone-sodium anaesthesia (40 mg/kg, i.p.) a stereotaxic lesion was made in the left lateral hypothalamic-mesencephalic junction, where the rostral part of substantia nigra and fibres

* A preliminary report of the present study was presented at the VII International Congress of the Collegium Internationale Neuro-Psychopharmacologicum in Prague, August 1970.

of the dopamine pathway are located (Zeman & Innes, 1963; König & Klippel, 1963; Hökfelt & Ungerstedt, 1969). The lesion was induced mechanically using a needle that had a semicircular tip to produce a spherical lesion with a diameter of 2 mm. By means of a stereotaxic apparatus the needle was lowered vertically into the position for the lesion, turned 360° and removed. The localization and extension of the lesion was verified microscopically in formaldehyde-fixed cresylviolet-stained brain slices (Nybäck, to be published). Sham lesions were made by lowering the needle to a position immediately above the dopamine pathway. It was not rotated before removing.

Fifteen days (chronic brain lesion) or 24 h (acute brain lesion) after the operation the rats were infused intravenously with [^{14}C]tyrosine (446 mCi/mmol, U.L. NEN Chemicals, 25 μCi/animal) for 20 min. The animals were killed immediately after the infusion and the striata of left and right hemispheres were dissected separately (Nybäck & Sedvall, 1969). Striata from three animals were pooled and homogenized in 0·4N $HClO_4$. After centrifugation, endogenous and radioactive tyrosine and dopamine were isolated from the supernatant fluid using chromatography on columns of alumina (Anton & Sayre, 1962), Dowex 50 WX 4 (Musacchio, Goldstein & others, 1966) and Amberlite CG 120 (Lewander & Jonsson, 1968). Endogenous tyrosine and dopamine were determined spectrophotofluorimetrically according to Wong, O'Flynn & Inouye (1964) and Carlsson & Waldeck (1958) respectively. Labelled tyrosine and dopamine were measured by liquid scintillation spectrometry, the efficiency of counting being 80 ± 5%. For details in the chemical procedure, see Nybäck & Sedvall (1970).

Animals with acute brain lesions were injected intraperitoneally with isotonic saline or chlorpromazine (Hibernal, Leo) (15 mg/kg) 40 min before the infusion of [^{14}C]tyrosine.

RESULTS

After about 3 h, when the animals had recovered from the anaesthesia, they exhibited a tendency to turn towards the side of the lesion. Apart from this the gross behaviour of the animals was normal.

Sham lesion. Fifteen days after the operation the animals were infused with [^{14}C]tyrosine and the contents of endogenous and labelled tyrosine and dopamine in left and right striatum were determined.

Table 1. *Contents of endogenous and labelled tyrosine* (Ty) *and dopamine* (DA) *in the striatum of unoperated and sham lesioned rats after i.v. infusion of* [^{14}C]*tyrosine.* Figures represent mean values of 4–6 determinations ± s.e.

	Ty (μg/g)	^{14}C-Ty (counts/min $g^{-1} \times 10^{-4}$)	DA (μg/g)	^{14}C-DA (counts/min g^{-1})
Unoperated rats				
left side	8·8 ±2·1	3·74 ±0·28	4·72 ±0·20	1650 ±126
right side	9·0 ±1·4	3·71 ±0·27	4·24 ±0·22	1550 ±135
Sham lesioned rats				
operated side	12·1 ±1·2	3·35 ±0·44	4·09 ±0·40	1350 ±138
unoperated side	12·1 ±1·0	3·39 ±0·29	4·21 ±0·40	1410 ±119

No significant differences were found in the levels of endogenous and labelled tyrosine and dopamine between the left and right striatum or between sham lesioned animals and unoperated controls (Table 1).

Chronic brain lesion. [^{14}C]Tyrosine was infused intravenously 15 days after the operation and the contents of endogenous and labelled tyrosine and dopamine in left and right striatum were determined.

The levels of endogenous and labelled tyrosine were about the same in both striata (Table 2). The contents of endogenous and labelled dopamine on the side of the lesion were only 13 and 15% respectively of the contents on the intact side. The specific activity of [^{14}C]dopamine, however, seemed to be higher on the lesion side than on the control side.

Table 2. *Contents of endogenous and labelled tyrosine (Ty) and dopamine (DA) in the striatum of rats with a chronic unilateral brain lesion after i.v. infusion of* [^{14}C] Figures represent mean values of 7 determinations ± s.e.

	Ty (μg/g)	^{14}C-Ty (counts/ min g^{-1})	^{14}C-Ty sp. act. (counts/ min μg^{-1})	DA (μg/g)	^{14}C-DA (counts/ min g^{-1})	^{14}C-DA sp. act. (counts/ min μg^{-1})
Control side ..	24 ±1·6	31 000 ±4400	1300 ±180	5·8 ±0·36	1600 ±110	290 ±27
Lesion side	30 ±2·0	33 000 ±4200	1100 ±110	0·74* ±0·16	260* ±54	390† ±57

* Differs from control side ($P < 0{\cdot}001$).
† Differs from control side ($P < 0{\cdot}05$).

Acute brain lesion. Twenty-four h after the operation the animals were injected with saline or chlorpromazine (15 mg/kg). Forty min later, [^{14}C]tyrosine was infused and the accumulation of [^{14}C]tyrosine and [^{14}C]dopamine in left and right striatum was determined.

Table 3. *Contents of endogenous and labelled tyrosine (Ty) and dopamine (DA) in the striatum of rats with an acute unilateral brain lesion after i.v. infusion of* [^{14}C]*tyrosine.* Figures represent mean values of 4–5 determinations ± s.e.

		Ty (μg/g)	^{14}C-Ty (counts/ min g^{-1})	^{14}C-Ty sp. act. (counts/ min μg^{-1})	DA (μg/g)	^{14}C-DA (counts/ min g^{-1})	^{14}C-DA sp. act. (counts/ min μg^{-1})
Saline	Control side	23 ±2·9	56 000 ±6000	2200 ±90	2·3 ±0·49	1600 ±440	760 ±140
	Lesion side	21 ±2·0	62 000* ±6500	3000* ±160	3·6 ±0·70	1600 ±340	450 ±34
CPZ	Control side	17 ±0·7	66 000 ±4300	3900† ±270	3·0 ±0·46	6100 ±870	2100† ±200
	Lesion side	21 ±2·1	71 000* ±3800	3600 ±480	3·6 ±0·72	2500‡ ±590	760‡ ±150

* Differs from control side ($P < 0{\cdot}05$).
† Differs from saline group ($P < 0{\cdot}01$).
‡ Differs from control side ($P < 0{\cdot}01$) but not from saline group ($P > 0{\cdot}05$).

The levels of endogenous tyrosine and dopamine were about the same in the striata of control and lesion side of both saline and chlorpromazine-treated animals (Table 3). The amount of [^{14}C]tyrosine was possibly higher on the side of the lesion than on the intact side in both saline and drug treated animals. On the control side the specific activity of [^{14}C]tyrosine was increased by chlorpromazine.

[^{14}C]Dopamine accumulated to about the same extent in both striata in the saline group. After chlorpromazine treatment the accumulation of [^{14}C]dopamine in the striatum of the intact side was markedly increased. However, in striatum on the side of the lesion the accumulation of [^{14}C]dopamine was not significantly increased by the drug. The specific activity of [^{14}C]dopamine was about the same in striata of saline-treated animals and on the lesion side after chlorpromazine treatment. In the striatum on the intact side of chlorpromazine-treated animals, however, the specific activity of [^{14}C]dopamine was markedly increased.

DISCUSSION

We have previously demonstrated that chlorpromazine accelerates the accumulation and disappearance of [^{14}C]dopamine formed from [^{14}C]tyrosine in the striatum of rats (Nybäck & Sedvall, 1969; Nybäck to be published). Other independent studies also indicate that chlorpromazine accelerates synthesis and turnover of brain dopamine. Thus accumulation of dopamine metabolites (Carlsson & Lindqvist, 1963; Andén & others, 1964; Bernheimer & Hornykiewicz, 1965) and disappearance of dopamine after tyrosine hydroxylase inhibition (Corrodi, Fuxe & Hökfelt, 1967; Neff & Costa, 1967) are accelerated by chlorpromazine treatment. Therefore it can be concluded that the increased accumulation of [^{14}C]dopamine in the striatum after chlorpromazine is due to, and can be used as an index of, accelerated dopamine synthesis in this brain region.

In the present study unilateral lesions were made in the lateral hypothalamic-mesencephalic junction where fibres of the nigro-neostriatal dopamine pathway are known to pass (Hökfelt & Ungerstedt, 1969). After the chronic lesion, but not after the sham lesion, there was a marked decrease in the contents of endogenous and labelled dopamine in the striatum ipsilateral to the lesion (Tables 1 and 2). This indicates that most fibres of the dopamine pathway were transected by the lesion with consequent degeneration of nerve terminals in the striatum. Degeneration of peripheral adrenergic nerves has previously been shown to result in a loss of both endogenous noradrenaline and the ability of the tissue to accumulate [^{14}C]noradrenaline formed from [^{14}C]tyrosine (Sedvall & others, 1968). In a recent study on monkeys with nigral lesions, Goldstein, Anagnoste & others (1969), obtained similar results.

The chlorpromazine-induced increase in [^{14}C]dopamine accumulation was almost completely abolished by the acute stereotaxic lesion in the lateral hypothalamic-mesencephalic junction (Table 3). The acute lesion did not significantly alter the ability of the striatum of saline-treated animals to form and accumulate [^{14}C]dopamine suggesting that the dopamine terminals were metabolically intact. The results strongly indicate that the acceleration of dopamine synthesis after chlorpromazine treatment is not due to a direct effect of the drug on dopamine nerve terminals in the striatum but requires intact nerve fibres running through the lateral hypothalamic-mesencephalic junction. Evidence for a similar mechanism regarding the effect of chlorpromazine on noradrenaline neurons in the spinal cord has been presented (Andén, Corrodi & others, 1967).

Although it is possible that the present lesion also affected other neurons which might influence dopamine synthesis in striatum, it seems most likely that the abolition of the chlorpromazine effect is due to transection of fibres of the nigro-neostriatal dopamine pathway. The results support the view that chlorpromazine accelerates dopamine synthesis in the striatum by an indirect mechanism, presumably by activating the nerve impulse flow in the nigro-neostriatal dopamine pathway.

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Antihypertensive and noradrenaline-depleting effects of guanethidine metabolites

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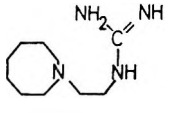
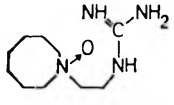
The antihypertensive and myocardial noradrenaline-depleting activities of the three identified guanethidine metabolites were compared with those of guanethidine itself. Metabolite A (guanethidine-*N*-oxide) and Metabolite B [2-(6-carboxylamino)ethylguanidine] both showed approximately 1/30th of the antihypertensive effect of guanethidine in rats. Metabolite A, but not Metabolite B, caused a depletion of cardiac noradrenaline stores. The intensity of this effect was between 1/10th and 1/30th that of guanethidine. Metabolite C [(6-carboxyhexyl)-2-iminoimidazolidine] was inactive on both parameters. [^{3}H]Noradrenaline uptake into isolated bovine nerve granules was not impaired by either guanethidine or its metabolites. It is concluded that the antihypertensive and myocardial noradrenaline depleting effects of guanethidine are produced by the unchanged drug rather than by one of the identified metabolites.

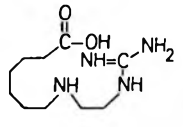
Guanethidine is widely used as an antihypertensive drug as well as a pharmacological research tool in the study of peripheral adrenergic mechanisms. Investigations have been made in the rat and in man on its excretion, distribution or metabolism or both (Dollery, Emslie-Smith & Milne, 1960; Bisson & Muscholl, 1962; Schanker & Morrison, 1965; Brodie, Chang & Costa, 1965; Furst, 1968; Rahn & Dayton, 1969). Only very recently, however, three metabolites have been identified from animal studies (McMartin, 1969) and their presence was demonstrated in the urine of hypertensive patients under guanethidine therapy (McMartin, Rondel & others, 1970). The structures of these metabolites are given below:



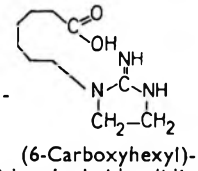
Guanethidine



Guanethidine *N*-oxide = Metabolite A



2-(6-Carboxyhexylamino-ethylguanidine = Metabolite B



(6-Carboxyhexyl)-2-imminoimidazolidine = Metabolite C

The present investigation was undertaken to determine the influence of these metabolites on arterial blood pressure of renal hypertensive rats and on noradrenaline storage mechanisms.

MATERIALS AND METHODS

Antihypertensive effects

Renal hypertension was produced in male rats, of 120–140 g, according to Goldblatt, by clamping the left renal arterial with silver clips (lumen 0·2 mm). Systolic blood pressure was measured under light ether anaesthesia by the plethysmographic method

of Wilson & Byrom (1939). The experiments started at least 5 weeks after operation, with groups of rats whose mean blood pressure ranged between 190 and 225 mm Hg. Guanethidine and its metabolites were injected subcutaneously once daily for 4 days. Blood pressure was measured 2 and 24 h after each of the first three injections and 2 h after the fourth injection.

Estimation of endogenous noradrenaline

The renal hypertensive rats were killed by decapitation 2·5–3 h after the last injection of guanethidine or a metabolite. In another series of experiments, normotensive male albino rats, 180–220 g, were treated with guanethidine or its *N*-oxide (metabolite A) and killed 24 h later. The hearts were homogenized in 10% trichloroacetic acid using a Polytron PT 20 OD homogenizer and centrifuged. Noradrenaline of the supernatant was adsorbed onto alumina at pH 8·6, eluted from it with 0·25 N HCl and estimated fluorometrically according to the trihydroxyindole procedure of Euler & Lishajko (1959), but using 10 N NaOH rather than 5 N NaOH for the formation of noradrenolutine (Anton & Sayre, 1962). The recovery of noradrenaline added to heart homogenates averaged 88·2% (n = 4). No correction for incomplete recovery has been made.

Estimation of [^{3}H]*noradrenaline uptake into isolated bovine splenic nerve granules*

Bovine splenic nerve granules were isolated by differential centrifugation with some modifications (Maître, Staehelin & Bein, 1970) of the methods described by Euler (1958) and by Schümann (1958). Briefly, the procedure consists of incubating suspensions of the granule fractions at 37° for 20 min in a medium containing 0·3 M sucrose, 0·009 M sodium phosphate buffer pH 6·8, 0·003 M ATP, 0·003 M $MgCl_2$ and 0·1 mM (±)-[^{3}H]noradrenaline (0·12 μCi/μg). After incubation, the granules were collected by passage of the suspensions through a HAWP 025 Millipore filter, dried and counted in a Packard Tri-Carb liquid scintillation spectrometer after addition of 10 ml of a 0·6% butyl-PBD (Scintillator CIBA) solution in toluene. Granule suspensions kept in an ice bath during incubation time were used as controls.

The (±)-noradrenaline-[1-^{3}H]* (6·5 Ci/mmol) was obtained from New England Nuclear, Boston, Mass., USA. It was diluted with cold (±)-noradrenaline (Fluka, Buchs, Switzerland). Guanethidine was used as sulphate. The metabolites of guanethidine were kindly put at our disposal by Dr. D. F. Elliott, CIBA Horsham.

Statistical significance of observed differences was analysed with the Student's *t*- test.

RESULTS

Antihypertensive effects

Guanethidine lowered blood pressure in a dose-dependent manner. For each dose the maximal effect was reached after the third injection. The blood pressure levels measured 24 h after the second and the third injection were still markedly lower than the initial values. This reflects the long duration of action of guanethidine (Table 1). The limited availability of the metabolites did not allow assay of more than one or two doses of each metabolite. Taking into consideration the dose-dependent antihypertensive effects of guanethidine, the results presented in Table 1

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

Table 1. *Antihypertensive effects of guanethidine and of three guanethidine metabolites in renal hypertensive rats.*

Substance and dose mg/kg, s.c.	Initial b.p. (mm Hg)	Change in blood pressure (mm Hg) 2 h after the 1st application	24 h after the 1st application	2 h after the 2nd application	24 h after the 2nd application	2 h after the 3rd application	24 h after the 3rd application	2 h after the 4th application
NaCl 0·9% (9)	212±3	− 3±3	− 6±4	−11±3	+ 1±2	−11±2	− 5±2	− 2±5
Guanethidine								
0·3 (4)	218±9				−28±6	−34±5	−19±4	−34±5
1 (4)	218±10				−33±1	−63±4	−38±2	−65±6
3 (12)	188±2	−34±6	−38±4	−64±4	−48±4	−70±4	−52±5	−69±5
6 (7)	198±6	−21±9	−35±6	−68±10	−56±10	−79±7	−74±15	−73±10
Metabolite A								
30 (4)	203±5	−31±4	− 8±7	−39±1	−28±10	−53±4	−35±7	−56±2
Metabolite B								
10 (4)	200±6	−25±5	− 3±1	−29±5	−21±10	−39±9	−13±13	−33±2
Metabolite C								
10 (4)	218±5	−16±4	− 8±4	−23±5	−14±10	−20±5	− 6±5	−11±6
30 (6)	226±6	− 8±2	− 2±3	−10±5	+ 3±2	−13±4	+ 3±2	−16±4

Substances were injected subcutaneously once daily for 4 days.
Blood pressure was measured plethysmographically under ether anaesthesia.
Figures represent mean values ± s.e.
( ) = number of rats.

indicate that metabolites A and B displayed antihypertensive activity, the intensity of which corresponded to approximately 1/30th of that of guanethidine. Metabolite C was inactive. The results further suggest that the duration of effect of metabolites A and B is of the same order of magnitude as that of guanethidine.

Effects on endogenous noradrenaline content

Renal hypertensive rats. The effects of a four days' treatment of renal hypertensive rats with guanethidine or its metabolites on noradrenaline content in heart and brain are shown in Table 2. Guanethidine, at a daily subcutaneous dose of 3 mg/kg, reduced myocardial noradrenaline content by about 80%. Among the three metabolites, only metabolite A (30 mg/kg each day) produced a depletion of endogenous noradrenaline in the heart. The intensity of depletion was of the same order of magnitude as that seen after guanethidine at a dosage 1/10th of that of metabolite A. Metabolites B and C were inactive. The noradrenaline content of brain was not altered by any substance.

Table 2. *Effects of guanethidine and metabolites on the content of endogenous noradrenaline in heart and brain of renal hypertensive rats.*

Treatment (mg/kg daily)	n	Noradrenaline μg/g heart	brain
NaCl 0·9%	7	0·76 ± 0·050	0·408 ± 0·022
Guanethidine 3	6	0·14 ± 0·015***	0·366 ± 0·031
Metabolite A 30	4	0·16 ± 0·018***	0·470 ± 0·024
Metabolite B 10	4	0·72 ± 0·032	0·396 ± 0·045
Metabolite C 10	4	0·77 ± 0·090	0·437 ± 0·032
Metabolite C 30	6	0·80 ± 0·041	0·424 ± 0·018

The substances were administered subcutaneously once daily for 4 days. The organs were removed 2·5–3 h after the last injection.
n = number of extracts.
*** = $P < 0.001$.
The rats whose blood pressure values are shown in Table 1 were used for these determinations.

Normotensive rats

For a better estimation of the relative depleting effects of guanethidine and metabolite A, normal rats were given a single injection of each and the cardiac noradrenaline content was determined 24 h later. The results show that metabolite A was at least 30 times less potent than guanethidine (Table 3).

Table 3. *Effect of a single dose of guanethidine or metabolite A on the noradrenaline content of the rat heart.*

Substance	mg/kg s.c.	n	Noradrenaline μg/g
NaCl 0·9 %		9	0·88 ± 0·047
Guanethidine	2	3	0·45 ± 0·068***
	6	3	0·25 ± 0·010***
	10	7	0·17 ± 0·021***
Metabolite A	20	3	0·81 ± 0·033N.S.
	60	3	0·64 ± 0·090*

The hearts were removed 24 h after treatment.
n = number of extracts.
N.S. = not significant ($P < 0·05$).
* = $0·01 < P < 0·05$.
*** = $P < 0·001$.

Effects on isolated nerve granules

Guanethidine inhibits the uptake of noradrenaline into sympathetic neurons *in vivo*. But it does not inhibit the uptake into isolated nerve granules *in vitro* (Maître & Staehelin, 1970). The effect of guanethidine *in vivo* might possibly be due to the action of a metabolite. It was therefore of primary interest to determine whether the known metabolites might impair the uptake mechanism *in vitro*. All three metabolites as well as guanethidine itself were unable to diminish significantly the uptake of [^{3}H]noradrenaline into isolated nerve granules up to concentrations as high as 10^{-3} M.

DISCUSSION

Metabolite A as well as guanethidine itself lowered arterial blood pressure on renal hypertensive rats and depleted myocardial noradrenaline stores with a potency which corresponded to less than 1/30th of that of guanethidine. Metabolite B produced a moderate antihypertensive effect. At a daily subcutaneous dosage of 10 mg/kg, its action was similar to that of 0·3 mg/kg daily of guanethidine subcutaneously (Table 1). The myocardial noradrenaline content was not diminished after repeated treatments with the mentioned doses of metabolite B (Table 2) whereas a four days' oral treatment with guanethidine (10 mg/kg daily) caused a 65% depletion of myocardial noradrenaline stores. It seems therefore that the influence of guanethidine and metabolite A on blood pressure and on catecholamine stores are not shared by metabolite B. Metabolite C did not show any activity on either parameter.

An inhibiting effect of guanethidine on noradrenaline uptake in adrenergic nerve granules has been proposed by Lindmar & Muscholl (1964). Although this view was substantiated by experiments *in vitro* made on ventricle slices (Shore & Giachetti, 1966) little is known about the exact effect of guanethidine itself on isolated granules. Studies on adrenomedullary granules showed that only very high concentrations of

guanethidine are able to inhibit noradrenaline uptake (Carlsson, Hillarp & Waldeck, 1963; Lundborg & Stitzel, 1968) and experiments on nerve granules failed to demonstrate an influence of the drug on the noradrenaline release rate even at concentrations of 10^{-3} M (Euler & Lishajko, 1962). In our experiments, guanethidine also failed to diminish the uptake of [^{3}H]noradrenaline into isolated splenic nerve granules up to a concentration of 10^{-3} M (Maître & Staehelin, 1970). Since *in vivo* effects of guanethidine might be due to its metabolites it was essential to determine the direct influence of the available metabolites on the noradrenaline uptake at the granular levels. But, as now shown, the three metabolites were as inactive as guanethidine itself. It is therefore unlikely that they are involved in such a mechanism.

Recent experiments on subcellular distribution of [^{3}H]guanethidine and metabolites in the rat heart showed that these metabolites were mainly present outside the granules. After treatment of the rats with guanethidine doses which completely normalized high blood pressure, the granule fraction contained only guanethidine (Maître & Staehelin, 1970). Therefore, it can be concluded that the antihypertensive effects as well as the noradrenaline depletion are due to unchanged guanethidine and not to any of its identified metabolites.

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The reversal of phenoxybenzamine-produced α-adrenoceptor blockade by the isomers of propranolol and INPEA

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Isomers of propranolol and *N*-isopropyl-*p*-nitrophenylethanolamine (INPEA) were used to demonstrate that there are two mechanisms by which the β-adrenoceptor blocking agents will reverse phenoxybenzamine-produced α-adrenoceptor blockade. In the seminal vesicle preparation, prior administration of either isomer initially protected the receptors from phenoxybenzamine blockade when the contact time for phenoxybenzamine was short. The isomers were equi-effective, suggesting that this action is independent of β-adrenoceptor blocking activity. When the contact time of phenoxybenzamine was prolonged, the ability of the isomers to protect the α-receptors was lost. In the rat blood pressure preparation, after the development of phenoxybenzamine-produced α-adrenoceptor blockade, (−)- and (±)-propranolol or (−)- and (±)-INPEA, in doses that produced marked β-adrenoceptor blocking activity, partially reversed the α-adrenoceptor blockade. Identical doses of (+)-propranolol or (+)-INPEA, which exhibited weak β-adrenoceptor blocking activity did not produce any reversal. The reversal of phenoxybenzamine-produced α-adrenoceptor blockade in this situation appears therefore to be dependent upon the development of β-adrenoceptor blockade.

The mechanism by which the β-adrenoceptor blocking agents reverse the α-adrenoceptor blocking actions of α-blocking agents has not been fully elucidated. Two hypotheses have been put forward. Gulati, Gokhale & Udwadia (1965), Olivares, Smith & Aronow (1967), Patil, Tye, & others (1968) and Guimaraes (1969) suggest that the β-adrenoceptor blocking agents compete with the α-adrenoceptor blocking agents for the α-receptor. On the other hand, Garrett, Malafaya-Baptista & Osswald (1965), Yamamura & Horita (1968) and Smith & Nash (1969) suggest that the reversal of α-adrenoceptor blockade by the β-adrenoceptor blocking agents is due to the blockade of β-receptors resulting in the unmasking of residual α-activity not previously blocked by the α-adrenoceptor blocking agent. This paper presents the results of the effects of the isomers of propranolol and of *N*-isopropyl-*p*-nitrophenylethanolamine on the reversal of phenoxybenzamine-produced α-adrenoceptor blockade and discusses the results in terms of the two hypotheses.

METHODS

Rat blood pressure preparation. Rats (Wistar strain), 160–220 g, were anaesthetized with urethane (100 mg/100g). The trachea was cannulated, the blood pressure monitored from the left carotid artery by means of a Condon manometer, which wrote on a smoked kymograph, and drugs were administered through a polythene

cannula inserted into the left femoral vein. The rats were heparinized with 50 units/100 g and drugs were administered in 0·1 ml volumes and washed through the cannula with 0·1 ml saline.

Reversal of α-adrenoceptor blockade. Responses to noradrenaline, phenylephrine or methoxamine at four dose levels were obtained. Phenoxybenzamine (2·5 mg/kg) was then administered slowly in 0·5 ml of saline and its effect allowed to develop over 60 min, at which time the responses to the agonist were again determined. (−)-, (+)- or (±)-Propranolol (1 mg/kg) or (−)-, (+)-, or (±)-INPEA (10 mg/kg) was then administered slowly in 0·25 ml saline and its effect allowed to develop for 10 min at which time the responses to the agonist were again noted.

β-Adrenoceptor blocking activity. Responses to isoprenaline at three dose levels were obtained. One of the isomers or the racemate of propranolol (1 mg/kg) or of INPEA (10 mg/kg) was administered and its effect allowed to develop for 10 min, at which time the effects of isoprenaline were again determined.

Guinea-pig seminal vesicle preparation

Seminal vesicles were taken from decapitated male guinea pigs (400–800 g) and suspended in a 10 ml organ bath containing Krebs solution gassed with 5% carbon dioxide in oxygen at 37°. The seminal vesicle was attached to a frontal writing lever which wrote on a smoked kymograph. The movements of the tissue were magnified 10 times and the tissue was subjected to a load of approximately 0·5 g. A vibrator was used throughout the experiment to ensure that the vesicle relaxed fully. Two kinds of experiment were made.

Effect of β-adrenoceptor blocking agents on α-adrenoceptor blockade, Responses to noradrenaline or phenylephrine at four dose levels were obtained, using a drug contact time of 1 min and a cycle time of 5 min, the preparation being washed twice between doses. Phenoxybenzamine was then applied to the tissue and allowed to act for 30 min, at which time the vesicle was washed twice and the responses to the agonist again observed. An isomer or the racemate of propranolol or INPEA was then added to the bathing medium and allowed to act for 15 min after which the preparation was washed for 15 min and the responses to the agonist again determined.

α-Adrenoceptor protection experiments. A four point dose response curve for noradrenaline or phenylephrine was obtained using a drug contact time of 1 min and a cycle time of 5 min. Phentolamine, or an isomer or the racemate of propranolol or INPEA was added to the bathing medium and allowed to act for 5 min, followed by phenoxybenzamine which was allowed to act for 5 or 30 min. The preparation was washed for 15 min and the responses to the agonist again determined. The results were compared with those of control experiments where the procedure was identical except that no β-adrenoceptor blocking agent or phentolamine was applied to the tissue.

Drugs and solutions

The drugs used were; phenylephrine hydrochloride; methoxamine hydrochloride Vasoxine; (−)-noradrenaline bitartrate; phenoxybenzamine hydrochloride; phentolamine methanesulphonate Rogitine; (−)-, (+), and (±)-propranolol hydro-

chloride and (−)-, (+)-, and (±)-INPEA- (*N*-isopropyl-*p*-nitrophenylethanolamine hydrochloride). All doses are quoted as the salt.

The composition of the Krebs solution was; NaCl 6·92; KCl 0·35; $CaCl_2$ 0·28; $NaHCO_3$ 2·10; NaH_3PO_4 0·16; $MgSO_4.7H_2O$ 0·29 and glucose 2·00 g/litre of distilled water.

RESULTS

Rat blood pressure

Reversal of α-adrenoceptor blockade. The inhibition of the pressor responses of 0·31 to 10·0 μg of noradrenaline, produced by 2·5 mg/kg of phenoxybenzamine were partially reversed by (−)- and (±)-propranolol (1 mg/kg) or (−)- and (±)-INPEA (10 mg/kg). (−)-Propranolol or (−)-INPEA were only slightly more effective than the corresponding racemate. No reversal was observed when the α-adrenoceptor blockade was challenged with (+)-propranolol (1 mg/kg) or (+)-INPEA (10 mg/kg). The results are in Fig. 1. The control responses before and after phenoxybenzamine were pooled. Phenoxybenzamine, 2·5 mg/kg almost abolished (90%) the pressor responses to the higher doses of noradrenaline, and propranolol was found to be more than 10 times more active than INPEA in its reversal of phenoxybenzamine-produced α-adrenoceptor blockade. The phenoxybenzamine-produced inhibition of the pressor responses to 1·25–20 μg of phenylephrine and 2·5–80 μg of methoxamine were not reversed by (−)- or (±)-propranolol or (−)- or (±)-INPEA.

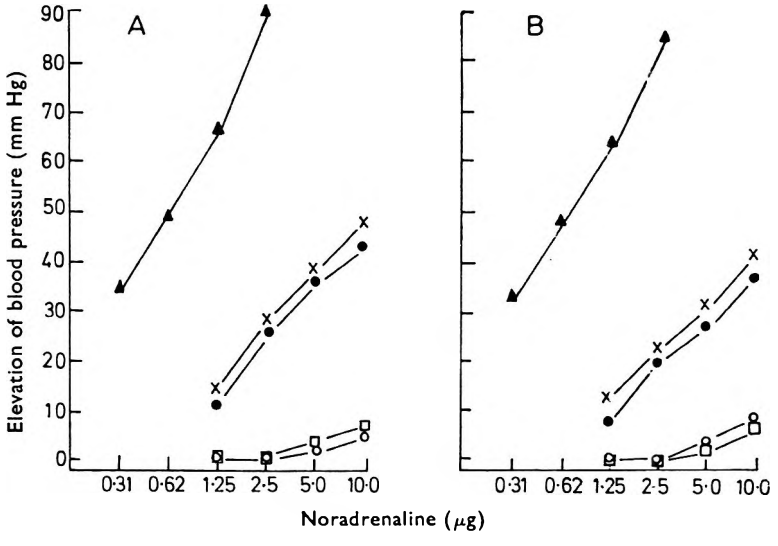

FIG. 1. The pressor responses of the rat blood pressure to noradrenaline in the propranolol series (A) and of the INPEA series (B) of experiments. ▲ — ▲ Control (noradrenaline alone); □ — □ Phenoxybenzamine; ○ — ○ Phenoxybenzamine with (+)-isomer; × — × Phenoxybenzamine with (−)-isomer; ● — ● Phenoxybenzamine with (±)-mixture. The doses were phenoxybenzamine 2·5 mg/kg; isomers and racemate of propranolol 1 mg/kg, and the isomers and racemate of INPEA, 10 mg/kg. Each point represents the mean of 6 determinations.

Before the administration of phenoxybenzamine, the resting blood pressure was within the range of 70–110 mm Hg and after the development of α-adrenoceptor blockade the blood pressure was lowered to 28–42 mm Hg where it stayed for the remainder of the experiment. In the presence of phenoxybenzamine, both the

isomers and racemates of propranolol and INPEA produced marked pressor effects, of 20–47 mm/Hg which were not well maintained, the blood pressure returning to the original level within 10 min.

β-Adrenoceptor blockade. The effects of the isomers and racemates of propranolol (1 mg/kg) and of INPEA (10 mg/kg) on the fall in blood pressure produced by 6·2–25 ng of isoprenaline are shown in Fig. 2. (−)-, and (±)-Propranolol or (−)-INPEA abolished and (±)-INPEA greatly reduced the response to isoprenaline but (+)-propranolol and (+)-INPEA had little effect upon the isoprenaline-produced fall in blood pressure.

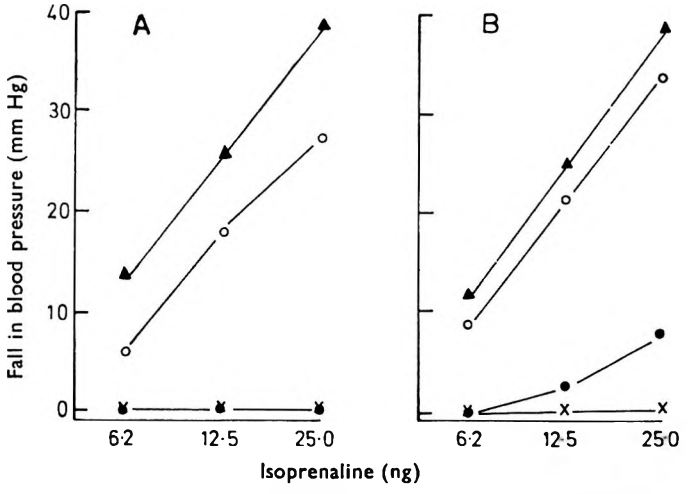

FIG. 2. The depressor responses of the rat blood pressure to isoprenaline in the propranolol series (A) and the INPEA series (B) of experiments. ▲ — ▲ Control (isoprenaline alone); ○ — ○ (+)-isomer; × — × (−)-isomer; ● — ● (±)-mixture. The doses were for the isomers and racemate of propranolol 1 mg/kg and for the isomers and racemate of INPEA 10 mg/kg. Each point represents the mean of 6 determinations.

Guinea-pig seminal vesicle preparation

Effect of β-adrenoceptor blocking agents on α-adrenoceptor blockade. Contractions of the seminal vesicle were obtained with 1·25–20 μg/ml of noradrenaline and 5·0–100 μg/ml of phenylephrine. There was a delay between the contract of the drug with the tissue and the tissue response which was most marked with the lower concentrations, lasting for as long as 25s. Phenoxybenzamine, 12·5 ng/ml, was allowed to act for 30 min and produced a marked reduction in the responses to noradrenaline and phenylephrine. The responses to the higher concentrations were reduced by more than 80%. The α-adrenoceptor blockade produced, persisted after repeated washings and challenge with agonist.

Neither isomer, nor the racemate of propranolol or INPEA in doses up to 100 μg/ml modified the responses of the phenoxybenzamine-treated seminal vesicles to the agonists. The isomers of propranolol or INPEA did not exhibit any intrinsic activity but in doses greater than 25 and 75 μg/ml respectively, they produced an inhibition of the response of the seminal vesicle to the agonist. This effect was readily reversed by washing the preparation with Krebs solution. Methoxamine was not used in these studies because in the concentrations of 2·5–10 μg/ml it produced persistent spontaneous activity.

α-Adrenoceptor protection experiments. Two contact times for phenoxybenzamine were employed, and at 5 min, 50 ng/ml of phenoxybenzamine produced a similar inhibition of the noradrenaline- or phenylephrine-produced contractions as did 12·5 ng/ml after 30 min. When the contact time of phenoxybenzamine was 5 min, the isomers or racemates of propranolol or INPEA, in concentrations of 25–100 μg/ml, or phentolamine, 0·1–2·0 μg/ml, applied to the tissue 5 min before the phenoxybenzamine, partially prevented the inhibition of the contractions of the seminal vesicle produced by phenylephrine (Fig. 3). The isomers were equi-effective in preventing the development of phenoxybenzamine α-adrenoceptor blockade and propranolol was no more effective than INPEA. However, when the contact time of phenoxybenzamine was 30 min, preparations pretreated with a β-adrenoceptor were not protected from the blocking action of phenoxybenzamine.

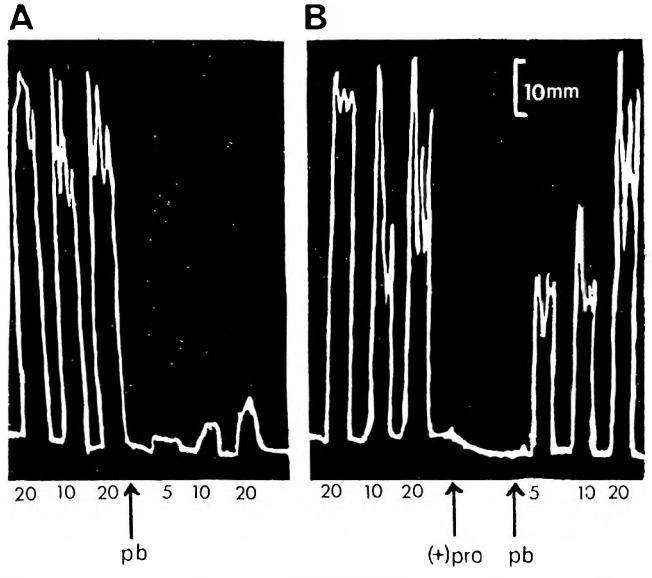

FIG. 3. Kymograph record of the responses of the guinea-pig seminal vesicle to noradrenaline, 5–20 μg/ml. Record A is a control showing the responses before and after phenoxybenzamine 50 ng/ml (pb). Record B shows the effect of (+)-propranolol 100 μg/ml (pro) administered 5 min before the phenoxybenzamine, on the phenoxybenzamine-produced inhibition of the noradrenaline-evoked responses. The contact time for phenoxybenzamine was 5 min.

DISCUSSION

In the urethane-anaesthetized rat, the inhibition of the pressor effects of noradrenaline produced by phenoxybenzamine were partially reversed by (−)- and (±)-propranolol and (−)- and (±)-INPEA. No reversal was observed with (+)-propranolol or (+)-INPEA. Phenoxybenzamine-produced inhibition of the pressor effects of phenylephrine—an amine with a marked α- and weak β-adrenoceptor activity—and methoxamine—an amine exhibiting only α-adrenoceptor activity—was not reversed by (−)- or (±)-propranolol or (−)- or (±)-INPEA. These observations are similar to those reported by Yamamura & Horita (1968) on the effects of (±)-propranolol on α-adrenoceptor blockade in the cat. Propranolol was more than 10 times more active than INPEA and in the doses employed, (−)- and (±)-propranolol and (−)- and (±)-INPEA abolished or greatly reduced the vasodepressor

effects of isoprenaline whilst the same dose of (+)-propranolol and (+)-INPEA exhibited very little β-adrenoceptor blocking activity (Howe & Shanks, 1966; Patil, 1968). That the phenoxybenzamine-produced α-adrenoceptor blockade is reversed by the β-adrenoceptor blocking agents only when the agonist is an amine with a significant β- as well as α- activity, and the isomer that exhibits little β-adrenoceptor blocking activity does not produce a reversal of α-adrenoceptor blockade, indicates that β-adrenoceptor blockade plays an important part in the reversal. The hypothesis that the inhibition of β-adrenoceptor activity results in the unmasking of residual α-adrenoceptor activity not blocked by the α-adrenoceptor blocking agent is therefore suggested by these observations. Both isomers and racemates of propranolol and of INPEA produced pressor responses in the presence of phenoxybenzamine. Similar observations have been reported for (±)-propranolol in the rat by Yamamoto & Sekiya (1969).

From the *in vitro* studies on the guinea-pig seminal vesicle preparation, it was found that prior administration of phentolamine or the isomers or racemates of propranolol or INPEA partially prevented the development of inhibition by phenoxybenzamine of the noradrenaline- and phenylephrine-produced contractions over 5 min. The isomers were equi-effective in preventing the development of α-adrenoceptor blockade and there was no difference in the effective concentrations of propranolol and INPEA. Patil & others (1968) observed similar activities of isomers of propranolol and INPEA in the vas deferens preparation in protecting α-adrenoceptors from dibenamine blockade. In the present experiments, when the duration of action of phenoxybenzamine was extended to 30 min, the administration of phentolamine or the isomers or racemates of propranolol or INPEA before or after the development of α-adrenoceptor blockade did not prevent the development of, or reverse the α-adrenoceptor blockade. The development of α-adrenoceptor blockade by phenoxybenzamine is complex. Initially the block is competitive and may be antagonized by catecholamines or competitive α-adrenoceptor blocking agents. When the block is complete, it is of a non-competitive nature and is unaffected by the presence of catecholamines or competitive α-adrenoceptor blocking agents (Nickerson & Gump, 1949; Furchgott, 1954). The inhibition of the development of phenoxybenzamine-produced α-adrenoceptor blockade seen in the seminal vesicle experiments cannot be mediated through the blockade of β-receptors, because the isomers are equi-effective in their protective activity and the seminal vesicle preparation exhibits only α-adrenoceptor activity (Guimaraes, 1969). These results support the hypothesis that the β-adrenoceptor blocking agents are capable of occupying and competing with phenoxybenzamine for the α-adrenoceptor. However, in view of the high concentrations required to produce this effect, the β-adrenoceptor blocking agents would appear to have a very weak activity at the α-receptor. Guimaraes (1969), using the guinea-pig seminal vesicle preparation, showed that (±)-pronethanol would protect the α-receptor from dibenamine blockade for a more than 20 min. Propranolol and INPEA did not afford protection of the α-receptors from phenoxybenzamine blockade when the contact time of the phenoxybenzamine exceeded 30 min. The differences in the duration of protection afforded by the β-adrenoceptor blocking agents from dibenamine and phenoxybenzamine α-adrenoceptor blockade may be explained by the fact that dibenamine has a slow onset of action (Nickerson & Goodman, 1947) and therefore a slower development of non-competitive α-adrenoceptor blockade. The inhibition of the responses of the seminal

vesicles to agonists produced by propranolol and INPEA is most probably a local anaesthetic action (see Davis, 1970).

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The oestrogenic and anti-oestrogenic properties of ring methyl-substituted stilboestrols

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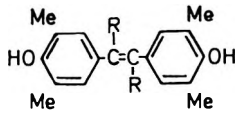
3,3′,5,5′-Tetramethylstilboestrol (I) and its α,α′-dimethyl- (II) and α,α′-diethyl- homologues (III) have been tested for oestrogenic and anti-oestrogenic activity. α,α′-Diethyl-3,3′,5,5′-tetramethylstilboestrol (III), by the uterine weight assay in immature mice, is $2{\cdot}1 \times 10^{-4}$ times as potent as an oestrogen as 17β-oestradiol [fiducial limits (0·95) = $1{\cdot}59 \times 10^{-4} - 2{\cdot}74 \times 10^{-4}$] but 3,3′,5,5′-tetramethylstilboestrol (I) and α,α′,3,3′,5,5′-hexamethylstilboestrol (II) were oestrogenically inactive at a dose of 0·8 mg. Compound III exhibited auto-inhibition of its own oestrogenic response at doses between 1·8 and 7·2 mg. None of the compounds tested inhibited the uterotrophic response to 17β-oestradiol. Compounds II and III, but not compound I, produced a highly significant inhibition of the vaginal cornification response to 17β-oestradiol when the test compounds (5 μg) and 17β-oestradiol were administered intravaginally in a single solution. It is suggested that the compounds II and III compete with 17β-oestradiol for the oestrogen receptor and that methyl groups positioned *ortho* to the phenolic hydroxyl group sterically interfere with the binding of the compounds to the oestrogen receptor, or with the initiation of the oestrogenic response, or both.

In the hope of producing anti-oestrogens which are effective when administered subcutaneously as well as locally, Clark & O'Donnell (1965a,b) synthesized and tested a series of ring methyl-substituted compounds related to diethylstilboestrol. Alkylation of the aromatic rings of di-*p*-hydroxyphenylalkanes and -alkenes, and ring A of 17β-oestradiol is known to reduce oestrogenic potency (Kaiser & Svarz, 1946; Shishido, Nozaki & Iwako, 1949; Niederl & Weiss, 1948; Iriarte & Ringold, 1958; Patton & Dmochowski, 1963), and reduced oestrogenic potency would allow a larger dose of the substance to be administered subcutaneously in tests for oestrogen antagonism. Clark & O'Donnell found that the introduction of four methyl groups, *ortho* to the phenolic hydroxyl groups in ψ-diethylstilboestrol, produced a compound with no oestrogenic activity in the Allen-Doisy test when 1 mg was given subcutaneously or 0·25 mg was given intravaginally to mice. No anti-oestrogenic activity was observed when the tetramethylated-ψ-diethylstilboestrol was administered subcutaneously but 25 μg inhibited the response to 17β-oestradiol when the two substances were administered intravaginally.

The observation by Emmens, Cox & Martin (1959) that in the α,α′-dialkylstilboestrols the highest anti-oestrogenic activity occurs in the dimethyl compound suggested that the examination of other ring tetramethylated stilboestrols would be of interest and we now report our studies with 3,3′,5,5′-tetramethylstilboestrol (I); α,α′,3,3′,5,5′-hexamethylstilboestrol (II); α,α′-diethyl-3,3′,5,5′-tetramethylstilboestrol (III) (Clark & O'Donnell, 1956b).



I R = H
II R = Me
III R = Et

METHODS

Preparation of solutions

(*a*) Solutions for subcutaneous injection were prepared immediately before each experiment. Aliquots of a stock ethanolic solution of 17β-oestradiol (1 mg/ml in 96% ethanol prepared every four weeks and stored at 4°) or volumes of a freshly prepared ethanolic solution of test substance (or both) were added to arachis oil to produce, after evaporation of the alcohol under nitrogen with slight warming on a water bath, the required dose of 17β-oestradiol or test compound in 0·15 ml arachis oil. Where systemic anti-oestrogenic properties of the compounds were to be examined, 17β-oestradiol and test compound were both added to the same volume of oil.

(*b*) Solutions for intravaginal injection were prepared in 2 or 4% aqueous Tween 80. Amounts of the ethanolic solutions of 17β-oestradiol or test compounds (or both) were added to undiluted Tween 80, and the alcohol evaporated off *in vacuo*, using a water pump. The solutions were then made up with warm distilled water to give the required doses in 0·02 ml of 2 or 4% aqueous Tween 80.

Uterine weight test

Oestrogenic properties of the compounds were assessed in randomly distributed groups of immature female mice (Tuck's No. 1 strain), each $\approx$12 g, injected subcutaneously with a range of doses of the compounds. The three-injection technique introduced by Rubin, Dorfman & others (1951) was used, each 'sub-dose' being given in 0·05 ml arachis oil at approximately 10 a.m. on each of three consecutive days. Animals were killed by cervical dislocation 24 h after the last injection and the uteri removed, freed of adhering connective tissue, and blotted before weighing on a Roller-Smith torsion balance. Actual uterine weights were used in calculations since no correlation between uterine weight and total body weight could be found.

Anti-oestrogenic activity was assessed by a comparison of the increase in uterine weight produced by two different doses of 17β-oestradiol alone with the same two doses of 17β-oestradiol each in combination with a range of non-oestrogenic doses of the test compounds.

Allen-Doisy test

Colonies of mice, ovariectomized at 6 weeks, were randomly distributed into groups and used at fortnightly intervals. Sensitivity was maintained by priming the mice every 6 weeks with 17β-oestradiol, 1 μg subcutaneously, in 0·05 ml arachis oil. An intravaginal two injection technique was used (Emmens & Cox, 1958) at approximately 10 a.m. on each of two consecutive days, each "sub-dose" being given in 0·01 ml of 2% aqueous Tween 80 for compounds II and III and a 4% solution for compound I because of its lower solubility. Vaginal smears were taken at approximately 10 a.m. and 5 p.m. on the 3rd day. Each mouse was scored as 0, 1 or 2 depending on whether neither, or one, or both smears indicated a positive oestrogenic response (Claringbold, 1956).

RESULTS

Uterine weight test

Neither test compound I nor II showed any oestrogenic activity at the doses tested (Table 1), which approached the limits of solubility, and which were much greater than the doses of 17β-oestradiol that produced an oestrogenic response. Compound III showed weak oestrogenic activity, being $2{\cdot}1 \times 10^{-4}$ times as potent as 17β-oestradiol [fiducial limits ($P = 0{\cdot}95$) $= 1{\cdot}59 \times 10^{-4} - 2{\cdot}74 \times 10^{-4}$]; at higher doses (0·9–7·2 mg) auto-inhibition was observed.

The compounds in non-oestrogenic doses were inactive in antagonizing the effects of concurrently administered 17β-oestradiol.

Table 1. *Oestrogenic properties of the test compounds, subcutaneously administered in the uterine weight test in immature mice.*

Expt. No.	No. of mice per group	Total dose of test compound/mouse (mg)	Total dose of 17β-oestradiol per mouse (μg)	Mean uterine weight ± s.e. (mg)
1	15	3,3′,5,5′-Tetramethyl stilboestrol (I)		
		0·1		7·98 ± 0·50
		0·2		8·12 ± 0·43
		0·4		8·50 ± 0·57
		0·8		9·66 ± 0·63
			0·03	20·00 ± 1·04
			0·06	26·56 ± 2·58
		0·0	0·0	8·24 ± 0·81
2	10	α,α′,3,3′,5,5′-Hexamethyl stilboestrol (II)		
		0·1		9·43 ± 1·08
		0·2		8·68 ± 1·01
		0·4		8·29 ± 1·13
		0·8		10·56 ± 0·71
			0·03	12·62 ± 1·13
			0·06	19·67 ± 1·74
		0·0	0·0	8·08 ± 0·81
3	10	α,β-Diethyl-3,3′,5,5′-tetramethyl-stilboestrol (III)		
		0·9		44·67 ± 1·79
		1·8		41·21 ± 3·00
		3·6		36·01 ± 2·83
		7·2		33·98 ± 1·59
			0·045	23·69 ± 1·14
			0·09	38·12 ± 2·10
4	16	0·12		19·97 ± 1·11
		0·16		26·72 ± 1·93
		0·21		33·78 ± 4·10
		0·28		40·11 ± 2·89
			0·03	26·04 ± 1·39
			0·06	37·01 ± 2·18

Allen-Doisy test

Intravaginal administration, to ovariectomized mice, of the compounds II or III (5 μg) together with 17β-oestradiol ($1{\cdot}5 \times 10^{-4}$–$13{\cdot}5 \times 10^{-4}$ μg) demonstrated a highly significant inhibition of the vaginal cornification normally produced by 17β-oestradiol ($P < 0{\cdot}01 > 0{\cdot}001$ and $< 0{\cdot}001$ for II and III, respectively). Compound I was inactive (Table 2).

Table 2. *Anti-oestrogenic properties of the test compounds given intravaginally together with 17β-oestradiol.* (Scores are totals for groups of mice: scoring system 0, no reaction; 1, one positive smear; 2, both smears positive)

Expt. No.	No. of mice per group	Total dose of test compound (μg)	Total dose of 17β-oestradiol per mouse (μg × 10^{-4}) 1·5	4·5	13·5
			score		
		Compound I			
1	12	0	13	20	22
		5	13	21	24
		Compound II			
2	10	0	4	9	13
		5	0	2	8
		Compound III			
3	10	0	10	13	17
		5	1	5	11

DISCUSSION

As was found for ψ-diethylstilboestrol (Clark & O'Donnell, 1965a) the introduction of four methyl groups *ortho* to the phenolic hydroxyl groups into α,α′-dimethylstilboestrol and α,α′-diethylstilboestrol has markedly reduced the oestrogenic potency. Thus there was no increase in uterine weight with compound II at a dose some 30 times the oestrogenic dose of α,α′-dimethylstilboestrol, and compound III was only weakly oestrogenic (approx. 1/5000 the potency of 17β-oestradiol). The negative regression of the uterine weights with larger doses of compound III suggests auto-inhibition and is reminiscent of the report that MER-25 is weakly uterotrophic at 0·2–1 mg dose but not with a dose of 5 mg (Lerner, Holthans Jr., & Thompson, 1958). Compared with the structurally isomeric 3,4-bis-(3,5-dimethyl-4-hydroxyphenyl)hex-2-ene which Clark & O'Donnell (1965a) found to be non-oestrogenic in mice at 1 mg s.c., there is clearly an increase in oestrogenic potency when the double bond is shifted to form the stilbene. This is similar to the difference in potency amongst the analogous isomers of diethylstilboestrol (Wessely & Kleedorfer, 1939).

In parallel with the increase in oestrogenic potency with the shift in position of the double bond, there is an increase in anti-oestrogenic potency, a similar level of inhibition of the response to 17β-oestradiol being obtained with 5 μg of compound III instead of 25 μg for 3,4-bis-(3,5-dimethyl-4-hydroxyphenyl)hex-2-ene (Clark & O'Donnell, 1965a).

The hoped for increase in anti-oestrogenic potency on replacing the α-ethyl groups present in compound III by α-methyl groups (compound II) has not been realized, both compounds producing a similar degree of inhibition at similar doses. Furthermore, compound II is less active as an oestrogen antagonist than the parent α,α′-dimethylstilboestrol which Emmens & others (1959) found to effectively inhibit the vaginal response to 17β-oestradiol at 0·2–0·4 μg.

With the complete absence of both oestrogenic and anti-oestrogenic activity in compound I it must be concluded that in this type of compound the same structural features that endow the molecules with oestrogenic activity are also needed for oestrogen antagonism. It seems therefore that compounds II and III are producing their effect by combining with the oestrogen receptor, but at the doses at which antagonism is observed they are unable to stimulate the normal oestrogenic response.

The "auto-inhibition" exhibited by compound III in the uterine weight test may be the result of general toxicity or may be interpreted as evidence of two-point attachment to the oestrogen receptor, high doses resulting in over-saturation of the receptor sites so that many molecules can gain access to only a single point of attachment.

Although dissociation constants for the test compounds have not been determined, by analogy one can deduce from a comparison of the pairs 2,6-xylenol ($pK_a = 10{\cdot}58$) and phenol ($pK_a = 9{\cdot}90$) (Wheland, Brownell & Mayo, 1948); and 2,4,6-trimethylphenol ($pK_a = 10{\cdot}88$) and *p*-cresol ($pK_a = 10{\cdot}19$) (Sprengling & Lewis, 1953) that the presence of the two *o*-methyl groups reduces the dissociation of phenols. The reduction in oestrogenic activity occasioned by *o*-methyl substitution may be due to this decrease in acidity with a resultant decrease in the strength of hydrogen bonds between the test compounds and the oestrogen receptor. Alternatively the methyl groups may, by increasing the bulk of the molecules, sterically interfere with the processes involved in binding and initiation of the oestrogenic response at the receptor site. That the oestrogen-receptor interaction is very susceptible to steric inhibition is suggested by the findings of Patton & Dmochowski (1963) who observed that the presence of a n-propyl group at position 2 in oestrone produced a 3500 fold decrease in oestrogenic potency, whereas a 2-methyl group produced only a 30 fold decrease.

It is possible that the presence of the 4 ring-methyl groups may affect the uptake and distribution of the test compounds from their sites of injection. That this is, however, not a primary reason for the absence of oestrogenic activity in compounds I and II and the low oestrogenicity of compound III is suggested by the fact that, on intravaginal administration, 5 μg of compounds II and III exhibit anti-oestrogenic responses, i.e. they are non-oestrogenic at this dose, whereas the median effective dose, as an oestrogen, for α,α'-diethylstilboestrol is $3{\cdot}7 \times 10^{-4}$ μg (Emmens, 1942).

CHEMISTRY

The 3,3′,5,5′-tetramethyl-α,α'-diethylstilboestrol used in the biological experiment was that described by Clark & O'Donnell (1965b). The α,α'-dimethyl homologue was prepared by an analogous series of reactions to those used for the diethyl compound, and the 3,3′,5,5′-tetramethylstilboestrol by an essentially similar process which differed only in the reduction of the 3,3′,5,5′-tetramethyldeoxyanisoin with lithium aluminium hydride before dehydration to the stilbene. That the 4,4′-dimethoxystilbenes possess the *trans* structure is indicated by their ultraviolet absorption spectra. The shape of the absorption curve for 4,4′-dimethoxy-3,3′,5,5′-tetramethylstilbene is similar to that given by Laarhoven, Nivard & Havinga (1960) for 4,4′-dimethoxystilbene and the expected decrease in the long wavelength absorbance with α,α'-alkylation is observed. The absorbances of the free phenols shows a similar dependence on α-carbon alkylation.

The change in the ultraviolet absorption spectrum of compound I in alcoholic solution stored for 7 days at room temperature stresses the need for fresh solutions to be used in experiments. The hypsochromic shift of the peak at 305 nm to 292 nm and the marked decrease in intensity of the band is consistent with the conversion of *trans*-stilbene into *cis*-stilbene [cf. *trans*-stilbene λ_{max} 295 (ϵ 27 000), cis-stilbene λ_{max} 280 (ϵ 13 500) nm]. Since *cis*-diethylstilboestrol is only very weakly active compared with its *trans* isomer such isomerization could be expected to be disadvantageous for oestrogenic or anti-oestrogenic activity.

EXPERIMENTAL

Infrared spectra were recorded on a Perkin-Elmer Infracord Spectrophotometer, model 137 or model 257, nmr spectra on a Varian A60 spectrometer and ultraviolet spectra on a Unicam SP700 or SP800A.

Microanalyses are by Mr. J. A. Stewart of the Microanalytical Laboratory, University of Leeds.

α-(3,5-*Dimethyl*-4-*methoxyphenyl*)*propionic acid.* Diethyl 3,5-dimethyl-4-methoxyphenyl malonate (63 g) was alkylated in the usual way using sodium (4·9 g), dry ethanol (80 ml) and methyl iodide (33 g) to yield diethyl (3,5-dimethyl-4-methoxyphenyl)-methylmalonate (47 g), b.p. 134–137°/0·15 mm. (Found: C, 66·4; H, 7·9. $C_{17}H_{24}O_5$ requires C, 66·2; H, 7·8%). ν_{max} (liq. film) 1730vs (COOR), 1600w, 1490s, 1470s, 1450sh (aromatic ring), 1250vs (aralkyl ether), 1160vs, 1110vs, 1020vs, 875m, 865m (isolated aromatic-H) cm^{-1}.

The derived ester was hydrolysed with 10% alcoholic KOH and the isolated malonic acid decarboxylated by heating at 170° for 30 min under water pump vacuum. Recrystallization of the residue from 40–60° light petroleum yielded the required α-(3,5-*dimethyl*-4-*methoxyphenyl*)*propionic acid* (23 g), m.p. 81–82·5°. Found: C, 68·95; H, 7·5. $C_{12}H_{16}O_3$ requires C, 69·2; H, 7·75%. ν_{max} (KCl disc) 1700vs (COOH), 1595w, 1490s, 1460s (aryl ring), 1415s, 1375m, 1330m, 1300s, 1280m–s, 1220vs (aralkyl ether), 1150s, 1010s, 955m, 930m (OH deformation), 885m (isolated aromatic-H) cm^{-1}.

α-(3,5-*Dimethyl*-4-*methoxyphenyl*)*propionyl chloride.* α-(3,5-Dimethyl-4-methoxyphenyl)propionic acid (24 g) and thionyl chloride (56 g) when heated together under reflux for 3 h yielded the required acid chloride (23·5 g), b.p. 150–152°/12 mm.

3,3′,5,5′-*Tetramethyl*-α-*methyldeoxyanisoin.* Anhydrous, powdered aluminium chloride (15·2 g) was added slowly with stirring to a cooled (<5°) solution of 2,6-dimethylanisole (21 g) in dry carbon disulphide (80 ml) at below 5°. α-(3,5-Dimethyl-4-methoxyphenyl)propionyl chloride (23·5 g) was then run in slowly, with stirring at below 5°. The flask was then allowed to warm to room temperature and heated on a water-bath for 2½ h. The mixture was poured into crushed ice containing a little hydrochloric acid and the oily layer taken into ether. Distillation of the ethereal extract yielded an oil (26·7 g), b.p. 170–182°/0·01 mm which slowly crystallized. Recrystallization yielded the *required ketone*, m.p. 67–68·5°. (Found C, 77·2; H, 7·8. $C_{21}H_{26}O_3$ requires C, 77·3; H, 8·0%.) ν_{max} (liq. film) 1678vs (aryl ketone), 1595s, 1485vs, 1450s (aryl ring), 1415s, 1372m, 1337s, 1295s (alkyl ketone), 1235–1222vs (aralkyl ether), 1152vs (aryl ketone), 1100m, 1047m, 1012vs, 954m, 926m, 908m, 882s (isolated aromatic H), 793s, 770m cm^{-1}.

2,3-*Bis*-(3,5-*dimethyl*-4-*methoxyphenyl*)*butan*-2-*ol.* 3,3′,5,5′-Tetramethyl-α-methyldeoxyanisoin (28·4 g) in dry ether (100 ml) was added slowly with stirring to a Grignard reagent prepared from methyl iodide (49 g), magnesium turnings (3·35g) and dry ether (200 ml). The mixture was then heated under reflux on a water bath for 2 h and left overnight. The reaction mixture was poured into ice and dilute hydrochloric acid, the ethereal layer separated and the aqueous layer extracted with ether. The combined ethereal solutions were washed with water, 10% sodium bicarbonate solution, 10% sodium thiosulphate solution, and water, and dried ($CaSO_4$). Distillation of the ether gave crystals (28·5 g) m.p. 104–121°. Fractional recrystallization of a portion (2 g) from 96% ethanol yielded erythro-2,3-*bis*-(3,5-

dimethyl-4-methoxyphenyl)butan-2-ol (1·12 g), m.p. 125–128°. (Found: C, 76·9; H, 8·8. $C_{22}H_{30}O_3$ requires C, 77·2; H, 8·8%). ν_{max} (KCl disc) 3500s (OH), 1600w, 1488vs, 1453s (aryl ring), 1370s, 1224vs (aryl ether), 1145vs (alkyl ether), 1129s, 1020s, 1000s, 883m, 875m (isolated aromatic-H), 770m, cm^{-1}. The threo *isomer* (0·21 g), m.p. 110–112° (Found: C, 77·5; H, 8·95%) was obtained by recrystallization from ethanol of the viscous residue obtained by evaporation of the mother liquors from the *erythro* isomer. The infrared spectrum differs from that of the *erythro* isomer only in the relative intensities of the peaks at 1453, 883 and 875 cm^{-1}.

4,4′-*Dimethoxy-α,α′,3,3′,5,5′-hexamethylstilbene.* The unrecrystallized mixture of diastereoisomers of 2,3-bis-(3,5-dimethyl-4-methoxyphenyl)butan-2-ol (5 g) was heated to 130° under an air reflux, iodine (50 mg) added to the melt and the mixture maintained at 130° for 1½ h. After cooling the mixture was dissolved in ether and the ethereal solution washed with 10% sodium thiosulphate solution and water, and dried ($CaSO_4$). Evaporation of the ether gave a sticky solid residue (4·5 g) which was crystallized repeatedly from light petroleum (60–80°) to yield the required 4,4′-*dimethoxy-α,α′,3,3′,5,5′-hexamethylstilbene* (1·42 g) m.p. 117·5–119°. (Found: C, 81·5; H, 8·55. $C_{22}H_{28}O_2$ requires C, 81·4; H, 8·7%.) ν_{max} (CCl_4) 1590w, 1488vs, 1449s (aryl ring), 1418m, 1375m, 1323s, 1230vs (aryl ether), 1188m, 1163vs (alkyl ether), 1116w, 1095m, 1078w, 1022vs, 937w, 883s (isolated aromatic-H) cm^{-1}. λ_{max} (ethanol) 212·5 (ϵ 31 000), 245 (ϵ 14 570) nm. Nmr: τ 8·18 (2 × CH_3–C; singlet); τ 7·73 (4 × CH_3–Ar; singlet); τ 6·28 (2 × CH_3OAr; singlet); τ 3·2 (Ar–H; singlet).

4,4′-*Dihydroxy-α,α′,3,3′,5,5′-hexamethylstilbene.* 4,4′-Dimethoxy-α,α′,3,3′,5,5′-hexamethylstilbene (2 g) in dry ether (20 ml) was added to a Grignard reagent prepared from methyl iodide (8·5 g), magnesium turnings (1·43 g) and dry ether (50 ml). The ether was distilled and the residue heated at 160–170° (bath temperature) for 3 h. The reaction mixture was cooled, decomposed with ice and 2N hydrochloric acid, and extracted with ether. The ethereal extract was washed with water, 10% sodium thiosulphate solution, and water, and dried ($MgSO_4$). Distillation of the ether gave a residue (1·8 g). Recrystallization from 60% aqueous ethanol gave the *required phenolic stilbene* (1·34 g), m.p. 219–222° (decomp.). (Found: C, 80·9; H, 8·0. $C_{20}H_{24}O_2$ requires C, 81·05; H, 8·15%.) ν_{max} (KCl disc): 3340vs (OH), 1605m, 1485vs, 1442s (aryl ring), 1412s, 1390s, 1376s, 1367s, 1360sh, 1319vs (phenol), 1269m, 1225vs (phenol), 1167vs, 1092s, 1074m, 1030m, 990m, 948m, 897m, 874s (isolated aromatic-H), 758s, 731 cm^{-1}. λ_{max} (ethanol): 212·5 (ϵ 30 510), 250 (ϵ 13 730), shoulder at *ca* 280 (ϵ *ca* 7100) nm.

3,3′,5,5′-*Tetramethyldeoxyanisoin* was prepared in a manner analogous to that for the α-methyl homologue, using 3,5-dimethyl-4-methoxyphenylacetyl chloride (9·1 g), 2,6-dimethylanisole (8·75 g), anhydrous aluminium chloride (5·7 g) and dry carbon disulphide (*ca* 50 ml). The *required ketone* was obtained as a very viscous oil (6 g) which slowly crystallized over many months, b.p. (bath temperature) 195–200°/4 × 10^{-3} mm. (Found: C, 76·7; H, 7·65. $C_{20}H_{24}O_3$ requires C, 76·9; H, 7·7%.) ν_{max} (liquid film): 1678vs (aryl ketone), 1600s, 1490vs, 1449s (aryl ring), 1414s, 1379m, 1340sh, 1318vs, 1227vs (aryl ether), 1181m, 1148vs (alkyl ether), 1064m, 1014vs, 900m, 876m (isolated aromatic-H), 797m, 769m cm^{-1}.

1,2-*Bis-(3,5-dimethyl-4-methoxyphenyl)ethanol.* 3,3′,5,5′-Tetramethyldeoxyanisoin (6 g) in ether (100 ml) was reduced with lithium aluminium hydride (0·18 g) in the usual way to yield a solid which was recrystallized from ethanol to yield the *required alcohol* (4·1 g), m.p. 134–135°. (Found: C, 76·1; H, 8·2. $C_{20}H_{36}O_3$ requires C, 76·4;

H, 8·3%.) ν_{max} (KCl disc): 3400s (OH), 1600w, 1486vs, 1460sh (aryl ring), 1437m, 1420m, 1376m, 1344m, 1318m, 1300m, 1263m, 1222vs (aryl ether), 1183m, 1144vs (alkyl ether), 1072s, 1012vs, 888m, 876s (isolated aromatic-H), 851m, 768m cm^{-1}.

4,4′-*Dimethoxy*-3,3′,5,5′-*tetramethylstilbene.* 1,2-Bis-(3,5-dimethyl-4-methoxyphenyl)ethanol (4 g), glacial acetic acid (30 ml) and concentrated hydrochloric acid (7·5 ml) were boiled under reflux for 10 min, diluted with water (50 ml) and the solid product filtered off. Recrystallization from benzene gave the *required stilbene* m.p. 219–220. (Found: C, 81·3; H, 8·25. $C_{20}H_{24}O_2$ requires C, 81·0; H, 8·15%.) λ_{max} (ethanol): 212·5 (ϵ 24 220), 237 (ϵ 19 620), 244·5 (ϵ 15 024), 305 (ϵ 27 360), 318 (ϵ 26 910), shoulders at *ca* 230 (ϵ *ca* 17 700), *ca* 295 (ϵ *ca* 24 700) and *ca* 330 (ϵ *ca* 18 160) nm. After storage of the alcoholic solution for 7 days at room temperature λ_{max}: 216 (ϵ 24 600), 234 (ϵ 23 920), 292 (ϵ 13 320) nm. ν_{max} (KCl disc): 1600w, 1590w, 1485vs, 1450s (aryl ring), 1305s (trans α,β-disubstituted ethylene), 1220vs (aryl ether), 996vs (oop deformations of *trans* disubstituted ethylene), 881s (isolated aromatic-H) cm^{-1}.

4,4′-*Dihydroxy*-3,3′,5,5′-*tetramethylstilbene.* 4,4′-Dimethoxy-3,3′,5,5′-tetramethylstilbene (2 g) was demethylated with methyl magnesium iodide [prepared from methyl iodide (10·6 g), magnesium (1·6 g) and ether] in the usual way. Fractional crystallization of the product from absolute ethanol yielded the *required phenolic stilbene* (1·2 g), m.p. 239–241°. (Found: C, 80·55; H, 7·45. $C_{18}H_{20}O_2$ requires C, 80·6; H, 7·5%.) λ_{max} (ethanol): 213·5 (ϵ 25 940), 238 (ϵ 16 840), 311 (ϵ 28 510), 327·5 (30 460), shoulders at *ca* 246 (ϵ *ca* 12 870), and *ca* 300 (ϵ *ca* 25 300) nm. λ_{max} (ethanol) after 7 days storage at room temperature: 216 (ϵ 24 600), 234 (ϵ 23 920), 292 (ϵ 13 320) nm. ν_{max} (KCl disc): 3380–3410vs (OH), 1605s, 1487vs (aryl ring), 1322vs (*trans* disubstituted ethylene), 1233vs, 1210vs (aryl ether), 955s (isolated aromatic-H) cm^{-1}.

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Identification and quantitative determination of some metabolites of methadone, isomethadone and normethadone

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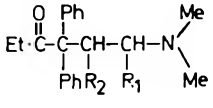
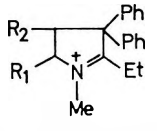
Isomethadone and normethadone are metabolized by microsomal preparations of guinea-pig liver to yield 2-ethyl-1,4-dimethyl-3,3-diphenyl-1-pyrroline and 2-ethyl-1-methyl-3,3-diphenyl-1-pyrroline respectively. The structures of the pyrrolines were established (by comparison with the pyrroline derived from methadone) by thin-layer chromatography and by infrared and nuclear magnetic resonance spectral data. Methadone, isomethadone and normethadone are also metabolized to the corresponding *N*-oxides. A gas chromatographic procedure for the quantitative determination of unchanged drugs, cyclic metabolites and *N*-oxides of methadone, isomethadone and normethadone in microsomal homogenates is described. The *N*-oxides were reduced before analysis.

Normethadone (Ic) has been reported by Vidic (1957), on the evidence of paper chromatography, to be *N*-demethylated in man to the corresponding primary and secondary amines, whilst Yoshida (1958) suggested that rats reduced normethadone to normethadol which was excreted partly as its glucuronide.

However, Beckett, Taylor & others (1968) established that methadone (Ia) is *N*-demethylated in man to a secondary amine which spontaneously rearranges to a pyrroline derivative (IIa) as the major product of metabolism.



Ia) methadone: R_1=Me, R_2=H
b) isomethadone: R_1=H, R_2=Me
c) normethadone: R_1=R_2=H



II a) pyrroline derived from methadone: R_1=Me, R_2=H
b) pyrroline derived from isomethadone: R_1=H, R_2=Me
c) pyrroline derived from normethadone: R_1=R_2=H

The structures of methadone, isomethadone and normethadone are similar, and it is therefore reasonable to assume that the major metabolites of isomethadone and normethadone would also be the corresponding pyrrolines (II).

N-Oxidation is now known to be a significant route of metabolism for some tertiary amines, e.g. propoxyphene (McMahon & Sullivan, 1964), imipramine (Fishman & Goldenberg, 1962), chlorpromazine (Beckett & Hewick, 1967) and chlorcyclizine (Kuntzman, Phillips & others, 1967).

Preliminary work in these laboratories indicated that methadone, isomethadone and normethadone gave *N*-oxides when incubated with hepatic microsomal preparations of some animal species. We have therefore investigated the structures of the

N-demethylated metabolites of isomethadone and normethadone, and developed a method for the quantitative analysis of the parent compounds (Ia, b and c), and their cyclic (IIa, b and c) and *N*-oxide metabolites, for each drug, in biological material.

METHODS

Thin-layer chromatography (*t.l.c.*). Glass plates 20 × 20 cm were spread to a thickness of 0·25 mm with a mixture of Silica Gel G (Merck) and water (1:2). The plates were first allowed to dry at room temperature (20°) for 10 min and then heated for 1 h at 110°. The solvent systems used are listed in Table 1. Dragendorff reagent was used to visualize the spots.

Table 1. *R_F values of methadone, isomethadone and normethadone and their cyclic and N-oxide metabolites on silica-gel plates, using different solvent systems.*

Solvent System	R_F values								
	M	Mc	Mo	I	Ic	Io	N	Nc	No
A	0·72	0·77	0·10	0·70	0·68	0·07	0·75	0·78	0·13
B	0·74	0·76	0·40	0·71	0·68	0·35	0·71	0·73	0·35
C	0·79	0·59	0·74	0·91	0·17	0·79	0·82	0·34	0·65

A = Chloroform–acetone–diethylamine (88:2:10).
B = Benzene–methanol–diethylamine (75:15:10).
C = Methanol–benzene–n-butanol–ammonia (0·88)–water (60:10:15:5:10).
M = Methadone, Mc = methadone cyclic metabolite, Mo = methadone *N*-oxide.
I = Isomethadone, Ic = isomethadone cyclic metabolite, Io = isomethadone *N*-oxide.
N = Normethadone, Nc = normethadone cyclic metabolite, No = normethadone *N*-oxide.

Gas-liquid chromatography (*g.l.c.*). A Perkin Elmer F11 chromatograph with a flame ionization detector was used. Chromatographic Column A was glass tubing $\frac{1}{4}$ inch o.d. and 2 m long, packed with acid washed, DMCS treated Gas Chrom Q (60–80 mesh) coated with OV17 (3% w/w). Column B was stainless steel tubing $\frac{1}{8}$ inch o.d. and 1 m long, packed with acid washed, DMCS treated chromosorb G (80–100 mesh) coated with Carbowax 6000 (2% w/w) and potassium hydroxide (5% w/w). The columns were kept for 24 h under their operating conditions before use, i.e. oven temperature 195° for Column A and 180° for Column B; injection block temperatures about 280°; hydrogen pressure, 20 lb/inch^2; air pressure, 25 lb/inch^2; nitrogen flow rate, 65 ml/min for column A and 36 ml/min for column B.

Isolation of the cyclic metabolites. Isomethadone (Ib) and normethadone (Ic) were each incubated at 37° with the 10 000 *g* microsomal supernatant preparation from male guinea-pig (Duncan Hartley, 400 g) liver homogenates which contained 1 g of liver in 2 ml isotonic KCl; each incubate contained 10 μmol of substrate, 4 μmol NADP, 20 μmol glucose-6-phosphate, 20 μmol $MgCl_2$, 60 μmol nicotinamide and 0·05M phosphate buffer, pH 7·4 in a total volume of 6 ml. The reaction was stopped after 80 min with 6N HCl (1 ml), and the precipitated proteins removed by centrifugation. The pH of each solution was adjusted to between 11 and 12 with sodium hydroxide (20%) and the solutes then extracted with redistilled diethylether (3 × 3 ml). The bulked ethereal extracts were concentrated to about 0·25 ml under reduced pressure. The cyclic metabolites were separated by preparative t.l.c. in the dark. Reference parent compounds were applied near the margins and solvent system C

(Table 1) was used for development. The plate margins were sprayed with Dragendorff reagent and the silica gel between the spots was collected in a glass stoppered centrifuge tube, distilled water (3 ml) and sodium hydroxide (0·2 ml) were added and the solution extracted with diethylether (3 × 3 ml). The ethereal solution was dried over anhydrous sodium sulphate, HCl gas passed, and the precipitated cyclic metabolite chlorides recrystallized from methanol.

The infrared spectra of these hydrochlorides as Nujol mulls were recorded using a Unicam SP200 infrared spectrometer.

The nmr spectra in D_2O were recorded using a Perkin Elmer R-10 nmr spectrometer plus a Northern Scientific 544 CAT with tetramethylsilane as the internal standard.

Isolation of the N-*oxides.* Methadone, isomethadone and normethadone were incubated with the 10 000 *g* liver homogenate described under 'isolation of the cyclic metabolites'. The solutions were extracted with chloroform and the concentrated chloroform extracts were applied to t.l.c. plates. Reference *N*-oxide spots were applied near the plate margins and solvent system B (Table 1) was used for the development. The plate margins were sprayed with Dragendorff reagent to visualize the reference spots and the silica gel between the spots corresponding to the *N*-oxides was collected and divided into two portions. Each portion was transferred to a glass-stoppered centrifuge tube. Distilled water (3 ml) and sodium hydroxide (0·2 ml) was added to one tube and the solution extracted with diethylether (3 × 3 ml). The ethereal solution was concentrated to about 50 μl and 2 μl was injected on to column A. To the other portion was added distilled water (3 ml), 6N HCl (0·3 ml) and 12·5% w/v $TiCl_3$ solution (0·2 ml, technical, Hopkin and Williams Ltd.) and the solution was stored at room temperature for 30 min. The solution was adjusted to pH 11–12, then extracted with diethylether and the solution concentrated and analysed on column A as above.

Synthesis of the N-*oxides* (*Ia, b and c*). The *N*-oxides of methadone, isomethadone and normethadone were prepared by the method published by Upjohn & Co. (1958) for the preparation of methadone *N*-oxide from methadone.

Stability of the tertiary amines (*Ia, b and c*) *to chemical reduction.* Methadone, isomethadone and normethadone (20 μg) as the hydrochlorides in distilled water (5 ml) were added separately to glass-stoppered centrifuge tubes; HCl (1 ml) and granular zinc (0·5 g) were added and the tubes then heated on a water bath at 60° for 0·25–3 h. At the end of the reaction, a solution (1 ml) of tripelannamine (10 μg) as internal standard was added to each tube and the solution adjusted to pH 11–12 with sodium hydroxide. The solution was extracted with ether and analysed by g.l.c. as above. The same procedure was repeated using $TiCl_3$ (0·2 ml) and HCl (0·3 ml) instead of granulated zinc and the solution stored in the dark at room temperature. The solution was extracted and analysed as above.

Quantitative analysis of the parent drug in the presence of its cyclic metabolite and N-*oxide.* Methadone (Ia), its cyclic metabolite (IIa) and its *N*-oxide (20 μg each) as the hydrochlorides in distilled water (4 ml) were placed in glass-stoppered centrifuge tubes together with tripelennamine (10 μg) as internal standard. Heat inactivated (10 000 *g*) liver homogenate (1 ml) was added. The solution was adjusted to pH 11–12 and extracted with diethylether (3 × 3 ml); the extract was concentrated and the unchanged drug and cyclic metabolite analysed using column A as above. The

extracted alkaline solution was made acid with HCl and the *N*-oxide reduced with $TiCl_3$ for 30 min as described above. The solution was then made alkaline, immediately extracted with ether and analysed as for parent drug. Retention times are in Table 2.

Table 2. *Retention times (min) of methadone, isomethadone and normethadone and their cyclic metabolites.*

	Methadone		Isomethadone		Normethadone	
Column	Unchanged drug	Cyclic metabolite	Unchanged drug	Cyclic metabolite	Unchanged drug	Cyclic metabolite
A	12·0	8·2	12·2	10·4	10·7	8·5
B	12·0	7·5	11·4	9·2	12·1	8·5

The same procedure was repeated with isomethadone (Ib) and normethadone (Ic) in the presence of their cyclic metabolites (IIb and c respectively) and their *N*-oxides.

N-*Demethylation of the tertiary amines* (*I b and c*). Isomethadone and normethadone (5 μmol each) were separately incubated with the 10 000 *g* liver homogenate previously described, to which semicarbazide (75 μmol in 1 ml of 0·05M phosphate buffer pH 7·4) had also been added. The reaction was stopped by the addition of $ZnSO_4$ (2 ml; 20%) and 2 ml saturated solution of $Ba(OH)_2$ and the precipitate removed by centrifugation. The amount of formaldehyde produced was determined by the method of Nash (1953).

RESULTS AND DISCUSSION

Incubation of isomethadone and normethadone with guinea-pig liver homogenate yielded formaldehyde which indicates that these compounds were *N*-demethylated in a manner similar to methadone.

Structure of the pyrroline metabolites. The ethereal extract of the solutions obtained from the isomethadone incubate yielded two spots on t.l.c. The R_F value of one spot corresponded with that of isomethadone, and the other spot gave R_F values in systems A and B that were similar to the R_F values obtained for the pyrroline derivative of methadone (see Table 1); t.l.c. was carried out in the dark since the metabolite was photosensitive.

Analysis by g.l.c. (Column A and B) of the ethereal extract of isomethadone gave two peaks, one of which corresponded to isomethadone whilst the other was of shorter retention time. The structure of the isomethadone metabolite giving the second spot or peak was proved to be the substituted pyrroline chloride (IIb) by the following evidence.

(i) The infrared spectrum of the chloride showed no $\overset{+}{N}$-H band corresponding to that of tertiary amine salt. (ii) The unusually high intensity of the adsorption band at 1665 cm^{-1} suggested a C=N stretching frequency rather than C=C. (iii) There was no absorption band at 1710 cm^{-1} corresponding to the carbonyl group in the parent compound. (iv) The nmr signal at 6·3τ gave integral for three protons which indicated one *N*-methyl substituent. (v) The triplet at 9·3τ indicated the presence of an endocyclic alkene, whereas the lack of signal at about 2·3τ indicated the absence

of an olefinic proton and thus the absence of the exocyclic double bond. (vi) No signal for vinyl hydrogen was obtained.

It is therefore concluded that metabolic monodemethylation followed by cyclization and dehydration occurs for isomethadone in a manner similar to that described for methadone (Beckett & others, 1968).

Similar ethereal extracts of the products of metabolism of normethadone also gave two spots on t.l.c. (and two peaks by g.l.c.), one of which corresponded to that of the unchanged drug and the other to the cyclic structure (IIc) as indicated by evidence similar to that presented for isomethadone. The double bond of the salt was again endocyclic (IIc).

Assay procedure for determining the tertiary amines and their metabolites. Synthetic methadone *N*-oxide was reduced quantitatively to methadone with $TiCl_3$/HCl. This treatment did not reduce methadone itself, nor did the presence of extracts from liver microsome fractions interfere. Similar results were obtained using isomethadone and normethadone. However, reduction with Zn/HCl was unsatisfactory as both the carbonyl and *N*-oxide were reduced. There was also some decomposition of the tertiary amines, when the reduction mixtures were stored after the acidic $TiCl_3$ was made alkaline.

Table 3. *Percentage recovery of methadone, isomethadone and normethadone and their corresponding cyclic and* N*-oxide metabolites after addition of each drug with its corresponding metabolites to heat-inactivated guinea-pig liver homogenate.*

Compound	Recovery		
	Unchanged drug	Cyclic metabolite	*N*-Oxide
Methadone	94·6	95·6	85·5
Isomethadone	96·4	97·5	86·2
Normethadone	94·0	98·4	91·2

A chloroform extract of a methadone incubation mixture gave spots on t.l.c. (solvent system A) the R_F values of which corresponded to those of synthetic methadone *N*-oxide, cyclic metabolite and methadone. The presence of the *N*-oxide was further confirmed by extraction from the t.l.c. plate followed by $TiCl_3$/HCl reduction to give methadone as confirmed by g.l.c. analysis.

Analysis by g.l.c. of an ethereal extract of the spot corresponding to *N*-oxide in the thin-layer chromatogram showed that methadone was absent before reduction. Ethereal extraction could therefore be used to remove unchanged methadone and its cyclic metabolite from an incubate to leave the *N*-oxide in the aqueous alkaline solution. Reduction of the *N*-oxide remaining in solution by the method described in the experimental section reformed methadone quantitatively, which was subsequently extracted and analysed by g.l.c. The product was analysed immediately after the solution was made alkaline because some decomposition of the tertiary amine ketones occurred under alkaline conditions. Similar results were obtained when isomethadone and normethadone were used.

A quantitative method for the analysis of unchanged methadone, its cyclic metabolite and its *N*-oxide was therefore developed as described in the experimental section. The method involved extracting the drug and its cyclic metabolite from an alkaline solution into ether and subjecting the extract to quantitative g.l.c. analysis using tripelennamine as internal standard. The *N*-oxide remaining in the alkaline solution was reduced with $TiCl_3$ after acidification with HCl to yield the parent amine which was extracted and analysed by g.l.c. The percentage recovery of these three compounds added to heat-inactivated liver homogenate is shown in Table 3. The method was also applied successfully to the analysis of isomethadone and its cyclic metabolite and *N*-oxide and to normethadone and its corresponding metabolites when present together in biological material.

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The distribution of methadone in man

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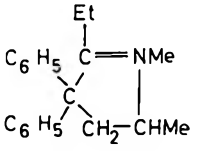
The distribution of methadone and its metabolite, 1,5-dimethyl-3,3-diphenyl-2-ethylidene pyrrolidine, in man, postmortem, is presented. Quantitative data for methadone and the metabolite in blood, bile, urine, liver, kidney, spleen, lung and brain samples show that methadone blood concentrations range from 0·22–3·04 μg/ml and are less than in bile and urine. The metabolite is found particularly in bile and urine. The liver and kidney concentrations are approximately equivalent unless the survival time is reduced by the presence of another CNS-depressant drug. Lung tissue is the richest source of methadone and brain the poorest. Chromatographic data for three other methadone metabolites are included.

Early urinary excretion studies of methadone in man (Scott & Chen, 1947; Cronheim & Ware, 1948; Way, Sung & McKelway, 1949) suffered from the analytical limitations of the solvent-soluble base-dye complex methods while the distribution studies of Alha & Ohela (1956) relied upon a non-specific colorimetric test together with a positive Straub reaction for measurement and characterization of the drug. In 1957, Vidic found methadone-derived primary and secondary amines in human urine after administration of the drug. An attempt by Pohland, Sullivan & Lee (1959) to synthesize *N*-desmethylmethadone resulted in the formation of 1,5-dimethyl-3,3-diphenyl-2-ethylidene pyrrolidine (I), which was isolated and identified by Beckett, Taylor & others (1968) as the major urinary excretion product of methadone in man.



I

In this paper the distribution of methadone and its major metabolite (referred to as metabolite 1) in man postmortem in relation to the cause of death, is described.

MATERIAL AND METHODS

Reagent grade chemicals were used. Methadone hydrochloride, benzhexol hydrochloride and lignocaine hydrochloride were of B.P. quality. A sample of metabolite 1 was made available by Dr. A. F. Casy. All tissues were deep frozen and all body fluids were refrigerated as soon as possible after collection.

The nature of the drugs present was established in each case by appropriate preliminary qualitative tests (see for example Clarke, 1969).

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Extraction procedure for methadone

Urine. To urine (10 ml) were added benzhexol hydrochloride solution (120 μg in 0·2 ml as internal standard) and 2N sodium hydroxide (1 ml). The mixture was then extracted with ether (2 × 20 ml) and the ether extracts evaporated in a tapered tube at 50° to 100 μl. Portions (3 μl) were used for g.l.c.

Blood. To blood (10 ml) were added benzhexol hydrochloride solution (30 μg in 0·05 ml as internal standard) and 2N sodium hydroxide (1 ml). The mixture was shaken with ether (2 × 20 ml). The ether solution was extracted with N sulphuric acid (10 ml) which was then made alkaline with 40% w/v sodium hydroxide solution (1·5 ml) and extracted further with ether (15 ml). The final ether extract was evaporated in a tapered tube at 50° to 100 μl and portions (3 μl) used for g.l.c.

Bile To bile (10 ml) were added benzhexol hydrochloride (120 μg in 0·2 ml) and 2N sodium hydroxide (1 ml). The mixture was shaken with ether (2 × 20 ml). The ether extracts were extracted as for blood. Portions (3 μl) from the final ether extract were used for g.l.c.

Tissues. Defrosted tissue (50 g) was homogenized without added fluid. Benzhexol hydrochloride solution (120 μg in 0·2 ml), 2N sodium hydroxide (10 ml) and distilled water (10 ml) were added to the homogenate and the mixture was extracted with ether (3 × 75 ml). The ether extracts were reduced to 100 ml by evaporation and then extracted with N sulphuric acid (40 ml). Sodium hydroxide (5 ml 40% w/v) solution was added to the acid and the whole was extracted with further ether (50 ml). This ether extract was evaporated in a tapered tube at 50° to 100 μl. Portions (3 μl) were used for g.l.c.

Gas-liquid chromatography (g.l.c.)

An F & M Biomedical 400 Gas Chromatograph fitted with a flame ionization detector was used.

Column 1. 3·8% W98 on Diatoport S 80–100 mesh packed in a four foot glass column of 3 mm i.d. Conditions: oven temperature 175°, flash heater temperature 255°, detector temperature 185°; nitrogen (carrier gas) pressure 40 lb/inch^2, flow rate 80 ml/min; air pressure 30 lb/inch^2; hydrogen pressure 25 lb/inch^2. The retention time for methadone was 5·4 min, for benzhexol 7·4 min and for metabolite 1, 3·7 min.

Column 2. 1% cyclohexane dimethanol succinate on Diatomite CQ, 100–120 mesh, packed in a 6 foot glass column of 3 mm i.d. Conditions: oven temperature 185°; flash heater temperature 290°; detector temperature 220°; nitrogen (carrier gas) pressure 40 lb/inch^2, flow rate 80 ml/min; air pressure 30 lb/inch^2; hydrogen pressure 25 lb/inch^2. The retention times were as follows: methadone, 4·6 min; metabolite 1, 6·9 min; lignocaine, 2·6 min; methaqualone, 13·3 min.

Quantitative analysis for methadone

Calibration curves using column 1 were prepared for the assay of blood samples using 30 μg of benzhexol hydrochloride for up to 50 μg methadone, and, for the assay of bile, urine and tissue samples, 1200 μg of the internal standard for up to 250 μg methadone. Linear relations were observed for these ranges and standards were included in every set of samples for assay.

Comparison of the small slightly impure sample of metabolite 1 with methadone showed equivalent response of the flame ionization detector for the two substances on a molar basis. Hence, calibration curves for methadone were applicable also to metabolite 1.

Column 2 was used when the preliminary tests indicated the presence of methaqualone. Lignocaine was a suitable internal standard: 30·5 μg of the hydrochloride was used for the blood samples and 102 μg for urine, bile and tissue homogenates, the range of methadone levels being as before.

Recoveries of methadone and metabolite 1 added to blank tissues and fluids were 87 and 88% respectively and of benzhexol 80% and lignocaine 89%.

Thin-layer chromatography (t.l.c.)

Silica gel G (E. Merck & Co.) was spread on glass plates in 0·25 mm layers which were dried at 110° for 1 h before use. Two solvents systems were used: (1) methanol–ammonia (s.g. 0·88) (100 : 1·5, by volume) and (2) benzene–dioxan–ethanol–ammonia (s.g. 0·88) (50 : 40 : 5 : 5, by volume). Solvent development was allowed to proceed for 10–12 cm from the point of sample application. After drying, the plates were examined under ultraviolet light (264 nm) before spraying for basic substances with Dragendorff reagent and then over-spraying with iodoplatinate solution.

RESULTS AND DISCUSSION

Qualitative data

Methadone and metabolite 1 were detected chromatographically in all extracts of urine. Both substances reacted with Dragendorff and iodoplatinate reagent on thin-layer chromatograms prepared using solvent 1; nicotine (derived from tobacco smoking) was usually present and the other unidentified bases thought to be derived from methadone administration, were also found in subjects 1, 2, 3, 4, 5, 10 and 11 (Table 1). The reactions of the Dragendorff-positive substances on the chromatograms to other reagents are shown in Table 1, which also includes the retention times

Table 1. *Chromatographic characteristics of methadone, metabolite* 1 *and other* **basic substances in urine extracts.*

Substance	Relative R_F value System 1	Relative R_F value System 2	Cis-aconitic anhydride	*p*-Nitroaniline/ Alkali	Ninhydrin	Relative retention time g.l.c. Column 1†
Methadone	1·00	1·00	Pink	Pink	Grey	1·00
Metabolite 1	0·35	0·95	—	Purple	Pink/mauve	0·69
Nicotine	1·54	0·66	Yellow	Pink	Pink	
Nicotine metabolite	0·50	0·55				
Metabolite 2	1·07	0·78	Pink	Red-black	—	1·2
Metabolite 3	0·80	0·10	—	Pink	Mauve	0·58
Metabolite 4	0·17	0·46	—	—	Mauve	

* i.e. Dragendorff-positive reacting substances, probably derived from methadone, referred to as metabolites 2, 3 and 4; no reactions were observed with Folin-Ciocalteau reagent, sodium nitroprusside, or reduced sodium nitroprusside (Ziegler & Pettit, 1964).

† Additional peaks present in extracts of blank urine specimens from smokers and non-smokers had relative retention times of 0·32, 0·38, 0·45 and 0·90.

of the substances after elution with ether from alkaline suspension of the silica gel "spot", concentration and chromatography on column 1, relative to methadone. From these data, it appears that metabolite 2 may contain a tertiary amino-group

because of its reaction with *cis*-aconitic anhydride, while metabolites 3 and 4 may each contain a primary amino-group (see Table 1). Vidic (1957) previously detected a primary amine in urine extracts after administration of methadone. Further identification of these metabolites, which appeared to be present in minor proportions to metabolite 1, was not attempted.

Quantitative data

The quantitative distribution of methadone and metabolite 1 in postmortem tissues from eleven male subjects is given in Table 2.

From Table 2 it may be seen that, except for subject 7, methadone was present in the blood; the absence of the metabolite from the blood samples may be a consequence of the assay method (limit of detection in a 10 ml sample is 5 μg). Methadone blood concentrations were always low compared with other specimens which may be an indication of protein binding in the tissues as demonstrated by Sung, Way & Scott (1953) in the rat.

The presence of methadone in the stomach content of subjects 2 and 4 is notable since recent injection sites were evident and oral administration of the drug was unlikely. Secretion of methadone in the gastric juice may account for these observations which are consistent with our previously unpublished finding of nicotine in the gastric content of smokers. This is not an unexpected observation in man in view of the work of Shore, Brodie & Hogben (1957) in animals.

Both the unchanged drug and metabolite 1 were found in specimens of bile and urine, the amount of the metabolite in the bile usually exceeding that of methadone whereas the reverse was found in the urine. Beckett (1969) reported the urinary excretion of 9·6% of an oral dose of methadone unchanged and 17·7% as metabolite 1 in 24 h, the proportion of methadone excreted in the urine ranging from 1–20% according to the controlled urinary pH value. It would appear from the results that the extent of biliary secretion at least of the metabolite 1 may indicate faecal excretion of a significant proportion of the drug in this form.

In the tissues both methadone and metabolite 1 are usually present with the exception of those subjects where a suspected acute overdose of methadone in the absence of tolerance is probable (subjects 9 and 10). Even so, the survival time might have been sufficient to allow formation and retention of the metabolite in some of the tissues.

The concentrations of methadone found in the tissues vary from one subject to another, probably reflecting individual tolerance and drug-taking habits. It is perhaps more informative to consider the relative tissue concentrations for each subject and these are shown in Table 3, the results being derived from Table 2 by relating each concentration to that of the liver taken as 1. This Table shows that liver and kidney levels are approximately equal except in subjects 9 and 10 in whom lack of tolerance or at least a reduced tolerance to the drug was probable. Also, the time interval between administration of methadone and death of subject 11 was shorter than cases in which methadone alone was the significant drug, i.e. the presence of a barbiturate or morphine as well as methadone decreased the survival time compared with an acute fatal overdose of methadone.

Methadone, as may be expected of a CNS-active drug, was found in the brain although in lesser amounts than in the other organs analysed. In three subjects (2, 4, and 8), metabolite 1 was also present.

Table 2. *Methadone and metabolite* 1 *concentrations*† *in body fluids and tissues from* 11 *male subjects.*

Subject No.	Age		Blood	Bile µg/ml	Urine	Liver	Kidney	Spleen µg/g	Lung	Brain	Stomach content mg	Other drugs	Circumstances
1	23	Methadone	2·13	8·9	19·3	19·2			24·0	2·2	7·0	Methylamphetamine (Tincture of Cannabis)	Probable acute oral overdose ? Loss of tolerance. About 100–200 mg 9 h before death.
		Metabolite 1	—	14·6	5·3	1·3			0·15	—			
2	22	Methadone	0·3	1·56	5·2	0·92	0·89	0·89	3·0	0·19	1·5	*Barbiturate (short-acting) morphine Amphetamine	? Overdose. Daily dose 10 mg.
		Metabolite 1	—	7·9	0·72	0·3	0·42	0·22	0·64	0·03	—		
3	22	Methadone	0·95	40·6	132	2·5	2·9	4·1	7·2	—	—	None	Survived 9 h after last dose.
		Metabolite 1	—	+	46·2	0·25	1·9	0·15	0·32	—	—		
4	19	Methadone	2·3	7·1	17·4	3·8	3·2	2·7	3·5	0·6	2·5	None	Probable acute overdose; possibly tolerant. Sur. 6 h.
		Metabolite 1	—	110	13·4	+	+	+	+	+	—		
5	27	Methadone	1·6	15·0	18·0	4·0	4·3	1·5	8·5	—	36·6	None	Took about 200 mg 16 h before death.
		Metabolite 1	—	102	9·2	0·41	0·16	0·04	0·98	—			
6	21	Methadone	1·2	10·2	5·0	3·7	3·32	3·8	8·7	2·0	—	Morphine	Had been taking 30 mg/day but not during last 3 days in hospital. Supplied by visitors?
		Metabolite 1	—	6·1	6·4	0·33	0·7	1·56	0·29	—	—	Methaqualone	
7	34	Methadone	—	—	0·52	0·58	0·67	1·56	—	—	—	Morphine	None given during 4 days in hospital before death.
		Metabolite 1	—	0·67	—	0·14	1·13	1·0	0·11	—	—		
8	28	Methadone	1·43	42·5	8·8	0·4	0·48	0·2	0·8	0·05	—	*Methaqualone	Oral methadone available; methaqualone prescribed.
		Metabolite 1	—	9·8	3·5	+	+	+	+	+	—	Alcohol	
9	32	Methadone	3·04	11·9	76·2	49·5	18·3	20·9	110	—	—	Methaqualone	Probable acute overdose; considered 'off drugs'.
		Metabolite 1	—	9·85	1·6	—	2·7	—	—	—	—		
10	19	Methadone	0·54	9·6	34·6	3·3	2·03	2·32	11·1	0·23	43	Amphetamine	Considered to have been 'off drugs'.
		Metabolite 1	—	0·2	4·5	0·8	0·63	—	—	—	—	Iprindole	
11	28	Methadone	0·22	1·9	5·5	0·25	0·14	0·18	1·35	—	—	*Morphine *Barbiturate (Intermediate-acting) Lymecycline	Took 30 mg per day probably with other drugs; survived about 5 h after barbiturate injection.
		Metabolite 1	—	17·7	1·65	0·13	0·17	0·40	0·07	—	—		

* Significant in respect of the cause of death.
† Since the internal standard was added to the specimens before extraction no correction factors have been applied to the results.

Table 3. *Ratios of tissue concentrations of methadone to the concentrations found in the liver, calculated on a wet weight basis.*

Subject No.	Liver concn μg/g	Liver	Ratio of concentrations to that of liver			
			Kidney	Spleen	Lung	Brain
1	19·2	1·0			1·25	0·12
2	0·92	1·0	0·97	0·97	3·26	0·21
3	2·5	1·0	1·16	1·64	2·88	
4	3·8	1·0	0·84	0·71	0·92	0·16
5	4·0	1·0	1·01	0·375	2·12	
6	3·7	1·0	0·89	1·025	2·35	0·54
7	0·52	1·0	1·12	1·29	3·0	
8	0·4	1·0	1·2	0·5	2·0	0·13
9	49·5	1·0	0·37	0·42	2·24	
10	3·3	1·0	0·61	0·70	3·36	0·07
11	0·25	1·0	0·56	0·71	5·4	

Lung tissue concentrations usually exceeded that of the liver (except subject 4). The finding of comparatively large amounts of methadone in the lungs compared with other organs correlates with animal work (Rickards, Boxer & Smith, 1950) and unpublished data (in the Department) for some other CNS-active drugs including tricyclic anti-depressants and phenothiazine derivatives. Although many organic bases may accumulate in the lungs of dosed animals, we have not always found a parallel in man. In toxicological analyses, when only qualitative results are needed, there are advantages in testing lung tissue in preference to liver especially since the extracts present fewer experimental difficulties.

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The binding of bupivacaine to maternal and foetal plasma proteins

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The binding of bupivacaine to maternal and foetal plasma proteins has been investigated using a three-compartment dialysis apparatus, in which maternal and foetal plasma could be examined simultaneously. Maternal protein bound approximately twice as much bupivacaine as foetal protein per gram of protein at drug concentrations in the range 0·05 to 5·0 μg/ml. Over the same range of concentrations, 92–78% of bupivacaine was bound to maternal plasma protein but only 35–31% was bound to human albumin indicating that other proteins are involved in the binding of the drug to plasma proteins.

We have investigated the maternal and foetal plasma concentrations at delivery of a number of drugs, local anaesthetic agents such as lignocaine and bupivacaine having been of particular interest (Thomas, Climie & Mather, 1968; Thomas, Climie & Mather, 1969; Thomas, Climie & others, 1969). A feature of these results is that, while there is some patient to patient variation, the average ratio of foetal : maternal plasma concentrations is invariably less than 1. For example in a series of 42 patients in labour, who received lignocaine epidurally, the average foetal : maternal plasma concentration ratio at delivery was 0·51 (range 0·33–0·89) (Thomas & others, 1969). Investigations with the local anaesthetic bupivacaine [1-butyl-2-(2,6-xylylcarbamoyl)-piperidine] have shown that the average foetal : maternal plasma concentration ratio at delivery is in the range 0·3–0·5 (Thomas & others, 1969; Reynolds & Taylor, 1970; Hollmen, Nummi & Ojala, 1970; Taylor, Reynolds & others, 1970). The data from these various investigations show that there is no relation between the time from administration of the drug to blood sampling (i.e. delivery) and the foetal : maternal plasma concentration ratio. This suggests that the rate of passage of these drugs across the placental barriers is relatively rapid. If this is so, in relation to the interval between administration of drug and delivery (possibly hours), it might be anticipated that the foetal : maternal plasma concentration ratio would be 1. The plasma concentrations which have been reported in all the investigations mentioned are total plasma concentrations. No attempt has been made to evaluate the proportion which is bound to plasma proteins. Since differential binding to maternal and foetal plasma proteins could be an important factor in controlling the foetal and maternal plasma concentrations of drugs, we have now investigated plasma protein binding of bupivacaine to both foetal and maternal plasma proteins. Reynolds & Taylor (1970) have stated that bupivacaine was bound to plasma proteins to the extent of 90–95% at concentrations occurring clinically. However, they presented no evidence to support this.

MATERIALS AND METHODS

Dialysis

Two types of all-glass dialysis apparatus were used. A two-compartment one when binding to one type of plasma was being investigated and a three-compartment one when binding to maternal and foetal plasma proteins were being examined simultaneously (Fig. 1). Each compartment had a capacity of 5 ml. The dialysis

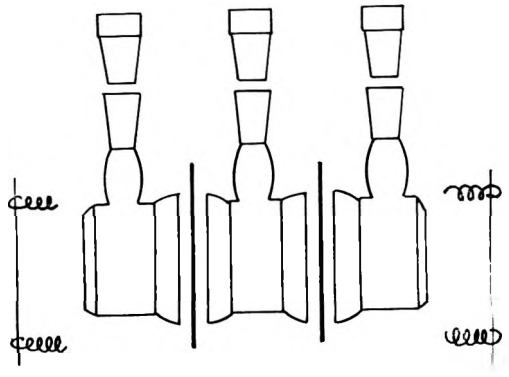

FIG. 1. Three-compartment dialysis apparatus used for simultaneous dialysis of maternal and foetal plasma against bupivacaine solutions. The capacity of each compartment is 5 ml. Construction is of glass with ground faces to support Visking membranes. Stainless steel springs hold the compartments together.

membrane was Visking dialysis cellulose which had been washed four times in distilled water at 80–90° and stored in flat sections at 4° until used. The apparatus was assembled and sterilized by autoclaving at 121° for 20 min. The compartments were filled aseptically under a laminar air flow hood. Dialysis was carried out at 37° for 42 h with constant shaking. One outer compartment of the dialysis apparatus was filled with foetal plasma, the other outer compartment with maternal plasma, while the centre compartment contained a solution of bupivacaine in buffer. Samples from the buffer compartment were checked regularly for protein using salicylsulphonic acid solution (20%).

Determination of bupivacaine

The gas chromatographic method of Thomas & others (1969) was used with one modification. A glass column, 3% OV-17 on 100–120 mesh Gas Chrom Q $\frac{1}{4}$ inch o.d., 6 ft long replaced that previously described. Nitrogen was the carrier gas at 37 ml/min. Under these conditions the retention times of bupivacaine and cyproheptadine (internal standard) were 9·0 min and 13·0 min respectively. A calibration curve of peak height ratio to weight of bupivacaine was constructed over the range 0·1–10·0 μg bupivacaine. The curve was a straight line and passed through the origin. Sample sizes from the dialysis solutions were selected to contain amounts of bupivacaine within this range.

Solutions

Bupivacaine hydrochloride was dissolved in Sørensen phosphate buffer M/15 adjusted to pH 7·35 at 25° (7·39 at 37°). The concentrations of the solutions ranged from 0·1 to 1000 μg/ml. These solutions were autoclaved at 121° for 20 min in 300 ml

bottles closed with a silicone elastomer multidose closure unit. No change in pH occurred during autoclaving.

A solution of human serum albumin (Commonwealth Serum Laboratories) in sterile buffer was prepared under aseptic conditions. Autoanalysis showed that the solution contained 4·8 g/100 ml total protein and 4·8 g/100 ml albumin. Cellulose acetate electrophoresis revealed the presence of $<1\%$ globulins.

Plasma

Blood was collected from 10 labour ward patients via the antecubital vein. Eight patients had received no medication during the previous 24 h, two had been given bupivacaine. Sodium citrate dihydrate was added to the collecting bottles to give a final concentration of approximately 5 mg/ml in the blood and the bottles were then sterilized. The blood was centrifuged at 2500 rev/min for 10 min and the plasma collected was pooled and sterilized by filtration through a Millipore filter (0·22 μm) with a fibreglass prefilter using positive pressure. After the patients had been delivered, foetal blood was collected from the placentae. Each placenta was supported in a large short stem funnel with the maternal surface uppermost and the cord protruding through the stem. A Braunula was inserted into the umbilical vein. The blood was removed from the placenta by connecting the Braunula to a sterile evacuated blood bottle containing sodium citrate dihydrate. Approximately 60 ml of blood could be collected in this manner. Plasma was separated from the whole blood by the same method as described for maternal plasma. The foetal plasma from the 10 patients was pooled and sterilized by filtration.

Plasma was also obtained from six volunteers each 36–38 weeks pregnant and kept separate. The albumin/total protein concentrations of these six plasma samples were normal and ranged (100 ml) from 3·4/6·4 g to 4·0/7·3 g (by autoanalysis).

Autoanalysis and cellulose acetate electrophoresis of the maternal and foetal plasma gave the results in Table 1.

Table 1. *Constituents of pooled maternal plasma and foetal plasma as determined by autoanalysis and electrophoresis.*

Constituent	Foetal g/100 ml	Maternal g/100 ml
Total protein (autoanalysis) ..	4·9	5·9
Albumin (,,) ..	3·0	3·1
Albumin (electrophoresis) ..	2·70	2·85
α1 Globulin (,,) ..	0·23	0·40
α2 Globulin (,,) ..	0·40	0·99
β-Globulin (,,) ..	0·70	0·76
γ-Globulin (,,) ..	0·87	0·92

RESULTS

The degree of binding of bupivacaine to plasma proteins from six patients varied from 71·3 to 91·6% over a total drug concentration range of 0·83 to 3·74 μg/ml. The degree of binding was related to the plasma concentration of drug (Table 2). Results from the three compartment apparatus are given in Table 3 from which it can be seen that bupivacaine is bound to a greater extent in maternal plasma than in foetal plasma. Also in Table 3 is the amount of bupivacaine bound per g of both maternal

Table 2. *Bupivacaine binding to diluted whole plasma.* Equilibrium dialysis used. Bupivacaine dissolved in Sørensen phosphate buffer M/15 pH 7·39 at 37°. Dialysis carried out at 37° for 42 h.

Plasma concentration (%) in protein compartment	% Bound	Bupivacaine concentration (μg/ml) Total	Free
100	81·2	3·2	0·6
50	66·0	3·1	1·0
25	47·8	2·7	1·4
12·5	36·4	2·5	1·6
6·3	17·7	2·3	1·9
3·1	7·1	2·1	1·9

Table 3. *The binding of bupivacaine to human maternal plasma proteins and foetal plasma proteins.* Equilibrium dialysis in a three compartment apparatus was used. Bupivacaine was dissolved in Sørensen phosphate buffer M/15 pH 7·39. Dialysis carried out at 37° for 42 h.

No. of exp.	Bupivacaine mean concn In plasma (μg/ml) Maternal	Foetal	In buffer μg/ml	Ratio maternal: foetal	Bupivacaine bound to plasma proteins Mean and range (%) (%) Maternal	Foetal	Mean (μg/g) Maternal	Foetal	Ratio maternal: foetal
4	0·13	0·05	0·01	2·40	92·1 (88·9–93·7)	81·1 (76·0–84·0)	1·98	0·87	2·30
3	0·15	0·08	0·02	1·85	85·5 (82·5–88·2)	73·2 (70·6–78·3)	2·23	1·23	1·81
3	0·26	0·11	0·04	2·31	83·5 (80·0–85·2)	61·9 (53·7–68·0)	3·69	1·42	2·59
2	0·49	0·25	0·09	1·93	80·7 (80·0–81·4)	63·2 (63·0–63·3)	6·69	3·27	2·04
3	4·47	2·30	1·00	1·94	77·6 (72·8–80·6)	56·5 (54·2–54·3)	58·8	26·5	2·21
3	35·1	14·0	9·2	2·51	73·8 (67·2–79·9)	34·3 (25·5–46·6)	439·0	97·9	4·48
2	81·4	30·4	21·0	2·68	74·2 (72·3–76·0)	30·9 (30·4–31·5)	1040	191·8	5·42
2	172·5	105·5	66·5	1·64	61·5 (60·7–62·3)	37·0 (35·5–38·6)	1796	796	2·25
3	1493	798	541	1·87	63·7	32·2	16135	5245	3·07

Table 4. *The binding of bupivacaine to human albumin as determined by equilibrium dialysis.* Bupivacaine dissolved in Sørensen phosphate buffer M/15 pH 7·39 at 37°. Albumin solution 4·8 g/100 ml. Dialysis at 37° for 42 h.

Bupivacaine Mean total concn (μg/ml)	Mean free concn (μg/ml)	Bound (% ± s.e.)	No. of determinations
0·037	0·024	35·1 ± 1·4	4
0·110	0·073	34·2 ± 1·4	8
0·234	0·156	33·5 ± 2·3	6
0·452	0·301	33·0 ± 2·1	5
4·52	3·11	31·0 ± 1·8	5
44·64	31·82	28·7 ± 1·2	5
224	165	26·3 ± 1·5	5
441	349	20·8 ± 0·9	6
787	658	16·3 ± 1·8	5

and foetal plasma proteins. These data show that maternal plasma proteins bind more bupivacaine than foetal plasma proteins over the range of concentrations of drug examined. Table 4 shows that while purified human albumin binds bupivacaine it does not do so to the same extent as total plasma proteins.

The effect of albumin concentration on the degree of binding of bupivacaine was investigated. It was found that bupivacaine was bound to the extent of 28·7, 11·8 and 6·0% when the albumin concentration was 4·8, 0·48 and 0·048 g/100 ml and the initial concentration of bupivacaine in the buffer solution was 78 μg/ml.

The effects of ions from the buffer solution on binding of bupivacaine to albumin was examined. In three separate dialysis experiments both the concentration of albumin (4·8 g/100 ml) and the amount of bupivacaine added (78 μg/ml) to the buffer were kept constant while the concentration of the phosphate buffer was varied. The three buffer concentrations used were 1/15, 1/30 and 1/60M and the respective degrees of binding were 28·7, 41·6 and 48·7%.

DISCUSSION

In establishing the procedures to be used a number of issues were considered. The first was the use of drug-free plasma for all the dialysis experiments. The drugs most frequently used in labour are analgesics of which pethidine is particularly popular. Most of these drugs are basic so the various drugs may compete for binding sites on the plasma protein. Hence data obtained for the degree of plasma protein binding of bupivacaine, when blood from patients who have received other drugs is used, could be suspect. To check this possibility, preliminary experiments were made in which the influence of pethidine on the binding of bupivacaine to human albumin was examined. Bupivacaine, 1 μg/ml in buffer, was bound to the extent of 33·0% when dialysed against albumin (4·8 g/100 ml), pethidine (5 μg/ml) added to the buffer reduced the binding to 23·5%. Because of the possibility of displacement of one drug by another from plasma proteins, only blood from the eight patients who had received no medication during the course of labour and the two who had received only bupivacaine was used.

A second point which was considered was the temperature of the dialysis. Dialyses at low temperatures reduce bacterial growth, however the amount of a drug bound to human albumin may be dependent on the temperature of dialysis (e.g. Sellers & Koch-Weser, 1969). While the temperature dependence of the binding of bupivacaine to proteins was not examined, it was considered preferable to dialyse at 37° in an apparatus designed to avoid contamination.

The degree of binding of bupivacaine to human albumin was found to be affected by the concentration of buffer solution. To check whether the buffer ions interfered with the relative binding of bupivacaine to maternal and foetal plasma proteins drug-free maternal plasma was dialysed against plasma from the placenta from the same patient. Bupivacaine was added to the maternal plasma and the system allowed to equilibrate. The ratio of concentrations of bupivacaine in the plasma samples was found to be between 2 and 2·5 (maternal : foetal). The amount of bupivacaine added was such that the plasma concentrations were in the range 0·4 to 3·4 μg/ml. These results obtained with systems in which no buffer was used were essentially the same as those obtained with the three compartment system (Table 3). This indicates that the buffer ions used did not significantly alter the relative binding capacities of maternal or foetal plasma proteins for bupivacaine. It would appear, therefore,

that the three compartment system is a reasonable *in vitro* model of the placenta to study the effects of plasma protein binding on placental transfer.

In the first four experiments the concentrations of bupivacaine in Table 3 were in the range commonly found clinically, in experiment 5 the total plasma concentration of 4·47 μg/ml is about the highest found in practice. The last four experiments were made to obtain information about the capacity of plasma proteins for bupivacaine. The results in Table 3 show that bupivacaine is extensively bound to plasma proteins over a wide range of concentrations. The data in Table 3 also show that the ratio of maternal : foetal plasma concentration of bupivacaine is around 2 over the range examined. The difference in concentration in the two plasmas could be due to differences in the degree of binding to the respective proteins or to different amounts of proteins present.

The ratios of bupivacaine bound to maternal and foetal proteins expressed in terms of μg bupivacaine per g of protein (from Table 1) for the first 5 experiments in Table 3 are approximately 2 which indicates that maternal plasma proteins bind bupivacaine more than foetal plasma proteins. This suggests that differential binding to maternal and foetal plasma protein plays a significant role in determining the transfer of bupivacaine across the placenta.

Binding of bupivacaine to human albumin

Albumin is considered to play a central role in binding of drugs to plasma proteins. Table 4 shows that while albumin is capable of binding bupivacaine over a wide range of concentrations, it does not bind the drug to the same extent as total plasma proteins. At clinical concentrations of bupivacaine (first five results), albumin binds approximately 30–35% of bupivacaine, while at similar concentrations of drug, whole maternal plasma proteins binds approximately 80–92%. The concentration of albumin in maternal plasma proteins was 3·1 g/100 ml while in the purified albumin system it was 4·8 g/100 ml. This provides further evidence that albumin does not account for the total binding of bupivacaine to plasma proteins. Treatment of the albumin binding data by the Scatchard method indicates that there are 1·16 statistical bupivacaine binding sites per molecule of albumin with an association constant of 552 litre/mol.

As other proteins must be involved in the binding of bupivacaine presumably there are a number of different sites each with different association constants and capacities to be considered when analysing the differential binding between the two kinds of plasma proteins. Plotting the data according to Rosenthal (1967) gives curves for both the maternal plasma and foetal plasma (Fig. 2) which indicates that more than one binding site is involved.

The difference in binding of bupivacaine to maternal and foetal plasma proteins could therefore be due to (i) the absence of a particular protein in the foetal plasma which had a high affinity for bupivacaine, (ii) the association constants of bupivacaine for the maternal and foetal proteins being different, or (iii) the number of sites available for binding being different.

The finding that bupivacaine binds more to maternal plasma protein than to foetal plasma protein is at variance with Goldstein, Aronow & Kalman (1969) who suggest that, in general, binding to maternal and foetal plasma is similar. However, Ganshorn & Kurz (1968) report that many drugs bind to maternal plasma protein more than they do to foetal plasma protein.

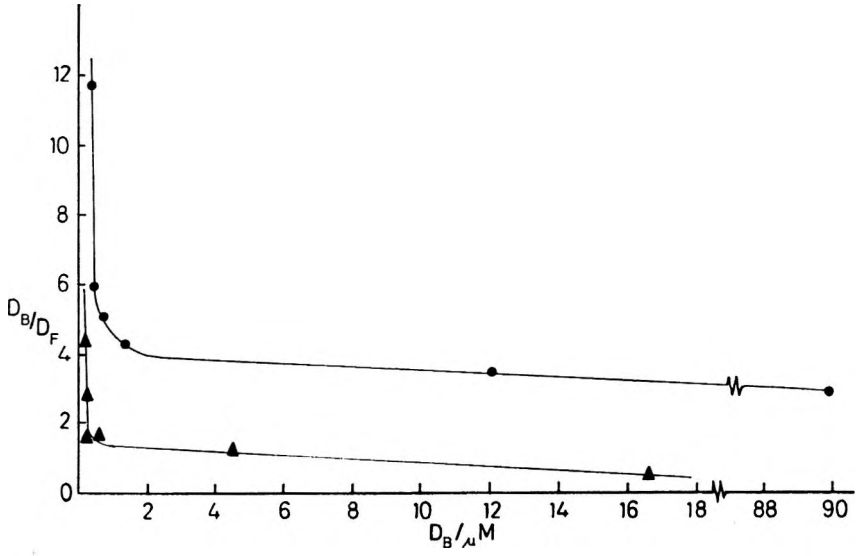

FIG. 2. Scatchard plot as modified by Rosenthal of the binding of bupivacaine to maternal plasma and foetal plasma. Temperature of dialysis 37°. Sørensen phosphate buffer M/15 pH 7·39. ●—● Maternal plasma. ▲—▲ Foetal plasma.

The observation that binding to human albumin accounts for less than half the plasma protein binding is contrary to the commonly held belief that this protein is the dominant one in binding and indicates that care should be taken in using albumin solutions as models for plasma protein binding studies.

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A note on the influence of diet in West Africa on urinary pH and excretion of amphetamine in man

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The urinary excretion of amphetamine was examined after the oral administration of (+)-amphetamine sulphate to two groups of subjects whose urinary pH fluctuated about mean acidic or alkaline values due to their different diets. The group with a balanced protein diet giving acidic urine, excreted much more drug unchanged than the group with a low protein diet giving alkaline urine. A small increase in protein intake in the group with alkaline urine made their urine pH acidic in a few days and increased their excretion of amphetamine to the same level as the group with acid urine.

The effect of diet on the urinary pH in man was reported by Wesley-Hadzija (1969). This work is now extended to examine the effect of different urinary pH values, arising from varying dietary customs, upon the excretion and reabsorption of amphetamine.

METHODS

Urine from males, 20–30 years, either on a balanced diet with adequate protein* (students; group 1) or on a low protein† diet (laboratory assistants; group 2) was collected every 2 h for 16 h starting at 7.0 a.m. on four successive days, and the pH of each collection was measured.

Additional proteins (one egg and half a pint of milk) were also given to three subjects of group 2 with their usual diet at 6.30 a.m. for four days and urine collection and measurement were continued for three succeeding days

An oral dose of 5 mg of amphetamine sulphate (3·7 mg amphetamine base) was administered in the morning of the fourth day to all subjects.

After amphetamine, the volume and pH of urine samples were measured and then those samples taken from 0–8 h and 8–16 h were separately pooled. The pH and volume of the pooled urine of each of several subjects from each group over a 36 h period, was also measured. pH measurements were usually made immediately after urine collections; a few samples were stored at 4°, and the pH measured within 24 h.

The amphetamine content of the urine was determined by gas liquid chromatography (Beckett & Rowland, 1965b).

* Diet: Group 1. Breakfast 6.30 a.m., coffee with milk (approx. 250 ml), bread (approx. 200 g) with butter or margarine (approx 30 g) and marmalade (approx. 30 g), rice or gari (150–200 g) boiled in water, fruit (approx. 100 g). Twice a week the fruit is replaced by one egg. Snack 10.00 a.m., coffee with milk (approx. 250 ml) and coffee cake (approx. 100 g). Lunch 1.00 p.m., meat (approx. 200 g) with vegetable and rice, beans or yam (approx. 200 g). Afternoon drink 4.30 p.m., squash (approx. 250 ml). Dinner 6.30 p.m., fish or meat (150–200 g) as stew with rice, yam, plantain or cassava (approx. 200 g), ice cream, fruit salad or cake (approx. 100 g) and cacao (approx. 250 ml).

† Diet: Group 2. Breakfast 6.30 a.m., gari, rice or corn (approx. 200 g) boiled in water. Lunch 1.00 p.m., groundnuts (approx. 50 g), 1–2 bananas, fried plantain or kenkey (approx. 200 g). Dinner 6.30 p.m., fish or meat (approx. 100 g) as stew and rice, yam, plantain or cassava as "fufu".

RESULTS

Amphetamine was recovered quantitatively from urine; other substances did not interfere with the assay.

The mean urinary pH values for each 2 h collection for 16 h on each of four days for the two groups of subjects were different (Wesley-Hadzija, 1969). Those of group 1 fluctuated about a mean of 5·9 (±0·56) and those of group 2 about 7·5 (±0·25); no overlap of the two groups occurred. None of the urine from group 1 subjects was more alkaline than pH 6·9 and all the samples from group 2 were more alkaline than this.

For the pooled urines collected over 36 h, the average pH value for group 1 subjects was 5·9 (range, 5·45–6·4) and for group 2 subjects it was 7·5 (range, 7·3–7·7). The ingestion of amphetamine did not alter the fluctuation and the range of pH observed in the two groups.

In group 2 subjects given additional protein, the urinary pH became acidic (≈pH 5) after the second breakfast with supplementary protein. Within a day of the supplementary protein diet being discontinued the urine pH rose to 6·7 or above.

In a separate experiment with six subjects of group 2, half a pint of milk at breakfast was sufficient to change the average pH of urine of the group to the acid side (Fig. 1).

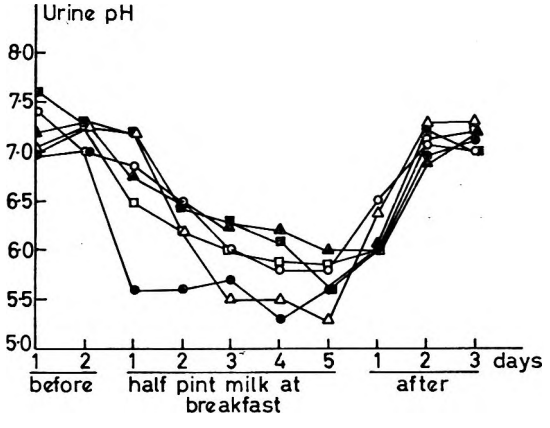

FIG. 1. Urine pH of pooled collections (7 a.m.–11 p.m.) of subjects on low protein diet—before (2 days), during administration of half a pint of milk at breakfast (5 days), and after return to low protein diet (3 days).

Group 1 subjects after an oral dose of 5 mg (+)-amphetamine sulphate excreted in their acidic urine 23 to 56% of unchanged amphetamine in 0 to 8 h and 5 to 13% from 8 to 16 h (Fig. 2).

Group 2 subjects after amphetamine excreted only 2 to 6% in 0 to 8 h and 0·5 to 3·0% in 8 to 16 h of unchanged drug after amphetamine administration (Fig. 2). Addition of protein to their diet for four days, at breakfast, resulted in acidic urine and the excretion of unchanged drug in amounts comparable to those obtained from group 1 subjects. Discontinuation of the protein supplement led to a reversion to alkaline pH and reduction in the amount of amphetamine excreted (Fig. 2).

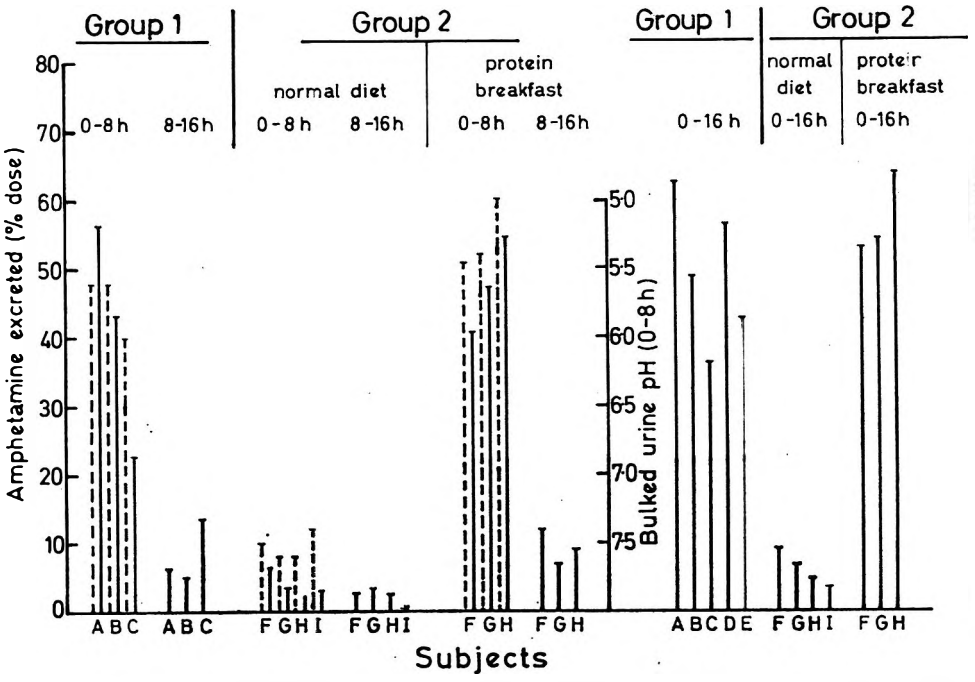

FIG. 2. Urinary excretion of amphetamine in students (Group 1) on balanced diet and also laboratory assistants (Group 2) on normal carbohydrate diet and then on a carbohydrate plus protein diet for four days. ——, Excretion of amphetamine; - - - - Urinary pH.

The two groups on different diets excreted amphetamine to different extents (Fig. 2) in a manner similar to that observed (Beckett & Rowland, 1964, 1965a) in excretion studies on subjects in U.K. whose urine was deliberately made acidic or alkaline by the oral administration of ammonium chloride or sodium bicarbonate respectively.

Thus diets can influence the excretion of amphetamine and therefore its biological half-life and duration of action in man. Since it has been shown that the excretion of many partially ionized drugs (Milne, Scribner & Crawford, 1958; Beckett & Brookes, 1969) and the ratios of metabolites to unchanged drugs (Beckett, 1969) are affected by making the urine acidic or alkaline with ammonium chloride and sodium bicarbonate respectively, it is probable that changes in diet which influence pH will have comparable effects.

Acknowledgement

The author thanks Prof. A. H. Beckett for advice and help.

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Decomposition of methoxamine in aqueous solution: identification of the decomposition products

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Combined gas-liquid chromatography-mass spectrometry is used to study the decomposition of methoxamine. The major breakdown product in aqueous solution under air is shown to be 2,5-dimethoxybenzaldehyde.

The mass spectrometer has been used to identify small quantities of medicinal substances, including sympathomimetics (Beckett, Tucker & Moffat, 1967; Reisch, Pagnucca & others, 1968; Reisch, Alfes & others, 1969), and the phenothiazines (Gilbert & Millard, 1969). Drugs and drug metabolites have also been identified using the mass spectrometer in combination with a gas chromatograph (Antony, Brooks & Middleditch, 1970; Capella & Horning, 1966; Gilbert, Millard & Powell, 1970).

We have used a gas chromatograph-mass spectrometer to identify the decomposition products of the sympathomimetic drug methoxamine hydrochloride B.P.C. [2-amino-1-(2,5-dimethoxyphenyl) propan-1-ol hydrochloride] which is unstable to light and heat (*Extra Pharmacopoeia*, 1967). The drug is often administered in aqueous solution and injections have to be sterilized by filtration or by autoclaving under nitrogen (B.P.C. 1968).

METHODS AND RESULTS

Apparatus

A Pye 104 gas chromatograph was used in conjunction with an A.E.I. MS 902 mass spectrometer. A Varian A-60A instrument was used for nmr spectrometry.

Degradation of sample. Methoxamine hydrochloride B.P.C. (5 mg) was dissolved in 0·1M borate buffer (2 ml) adjusted to pH 9, or 0·1M phosphate buffer (2 ml) adjusted to pH 6. These solutions were sealed in 10 ml clear glass ampoules to ensure excess oxygen was available and then stored at 80° in a constant temperature bath for 24 h and 1 week respectively.

Thin-layer chromatography. Thin-layer chromatograms were on Silica Gel G (Merck) layers, 300 μm thick. Developing solvent was the organic layer separated from n-butanol–acetic acid–water (5 : 1 : 4). Spots were detected by fluorescence under ultraviolet light (long wave), and by spraying with 0·2% ninhydrin in n-butanol, with colour development for 5 min at 110°.

Methoxamine (R_F 0·59) did not fluoresce but produced an intense red spot with the ninhydrin reagent. The degraded solution contained one main oxidation product (R_F 0·84), which fluoresced strongly under ultraviolet light; no colour developed after treatment with the ninhydrin reagent.

Gas-liquid chromatography–mass spectrometry. The column consisted of 2% SE-52 on Embacel (M & B Kieselguhr) packed in a 5 ft stainless steel column (¼ inch o.d.), column temperature 150°, inlet port temperature 250°, and a helium flow rate of 50 ml/min at 10 psi. The molecular separator, which worked on the opposed jet principle (Ryhage type) was contained within the g.l.c. oven. The inlet line to the mass spectrometer source was kept at 200°. The mass spectrometer had a resolving power of 1000 (10% valley definition) and was running at a source temperature of 220° and a beam energy of 70 eV. The total ion current monitor was used to record the presence of any material passing through the system.

The degraded solution was evaporated to dryness and then dissolved in 0·5 ml of methanol AR before injection into this system. An amount (5 μl) of this solution was injected into the heated inlet port and under the conditions described, one peak was recorded after 4·3 min. The spectrum of this compound was recorded (Fig. 1) as was the background spectrum. On raising the column temperature a second peak appeared; this was the remaining unchanged methoxamine. Both degraded samples of methoxamine produced this same peak, although less degradation product was formed at pH 6.

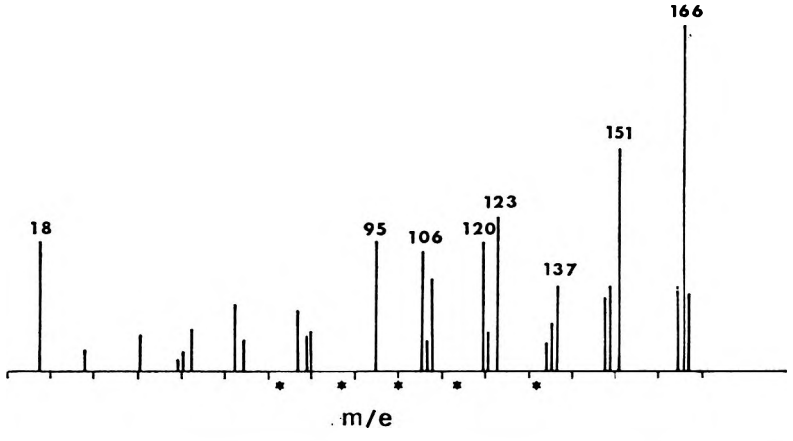


FIG. 1. Mass spectrum of the degradation product. Stars indicate position of metastable peaks.

Mass spectrum of the oxidation product. The molecular ion appearing at m/e of 166, was also the base peak in the spectrum, reflecting the relative stability of aromatic compounds, and corresponded to the aldehyde produced by oxidation of the alcoholic group with a subsequent loss of the amine side-chain. A fragmentation pattern (Fig. 2) for 2,5-dimethoxybenzaldehyde was derived which accounted for all the main peaks and metastable peaks observed. The spectrum was thus evidence for the formation of 2,5-dimethoxybenzaldehyde during the oxidation of aqueous solutions of methoxamine.

A commercial sample of this aldehyde was not available so a larger quantity was degraded, the aldehyde extracted and the nuclear magnetic resonance spectrum measured as conclusive evidence.

Preparation of sample for nmr determination. Methoxamine hydrochloride (100 mg) was degraded as described and the aldehyde separated by passing the solution through a cationic ion-exchange resin packed in a 2 ft chromatography

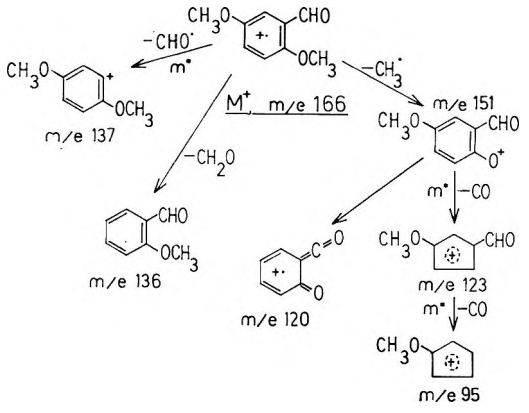


FIG. 2. Fragmentation pattern for 2,5-dimethoxybenzaldehyde.

column. The resin used was Zeokarb '225' 1% D.V.B. 50–100# (Permutit Ltd.). Intact methoxamine and other basic materials were retained on the resin, the aldehyde was then extracted from the eluent with diethyl ether in the presence of excess sodium hydroxide. The solvent was evaporated under reduced pressure, the sample was then dried at 50° for 2 h at 10 mm Hg. This sample produced one spot on the t.l.c. system at R_F 0·84. The melting point was 52° and that of the semicarbazone 208° (Dictionary of Organic Compounds, Vol. II, p. 1053, gives 53° and 208° respectively).

Nmr spectrum of the degradation product (Fig. 3). Chemical shifts were measured as τ values, using tetramethylsilane as an internal reference. The solvent was deuterochloroform, a peak, τ −0·43 (shown as one proton on the integrated curve)

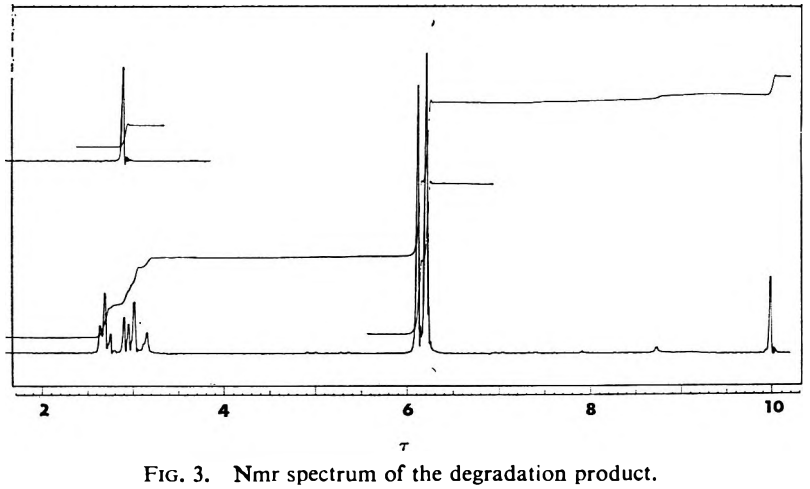


FIG. 3. Nmr spectrum of the degradation product.

is typical of an aldehyde proton resonance, while singlets at τ 6·12 and τ 6·22 represent the two methoxy groups (3 protons each), the aromatic resonance appears in the region τ 3·0 as expected (3 protons on integration). The position of these resonance peaks and the absence of any other proton resonance is confirmation that the major degradation product is a dimethoxybenzaldehyde.

Other oxidation products. A process resulting in the formation of this aldehyde would also be expected to produce acetaldehyde and ammonia, the former substance being itself oxidized to acetic acid. Using suitable t.l.c. and g.l.c. systems, acetaldehyde was not detected in the degraded sample.

A small quantity of acetic acid present in an aqueous solution containing several interfering substances is difficult to isolate, and the g.l.c. method of Emery & Keomer (1961), was used. This consisted of a 3 ft glass column containing 20% Tween 80 on acid-washed, silanized Chromosorb W 80–100#, maintained at a column temperature of 108°, injection port temperature 200° and a carrier gas flow rate of 20 ml/min at 5 p.s.i. This system easily detected 1 μl of a 0·1% solution of acetic acid, the retention time being 6·1 min. A sample of the degraded solution was acidified (using hydrochloric acid) to liberate the free acid and then chromatographed (Fig. 4).

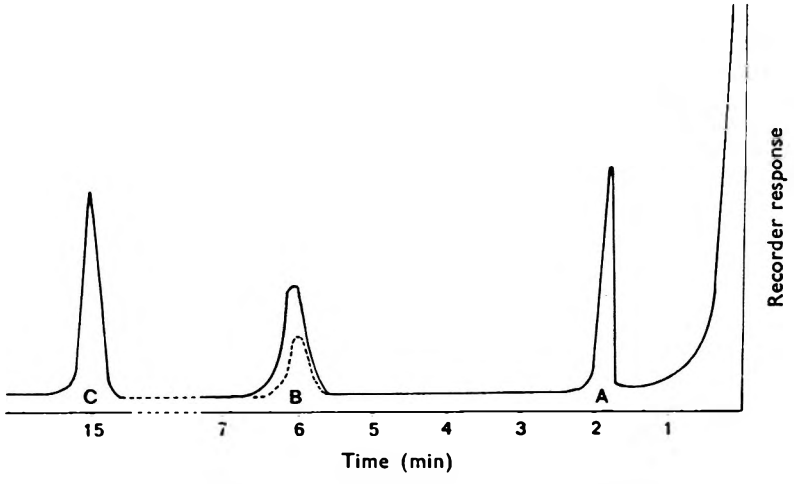
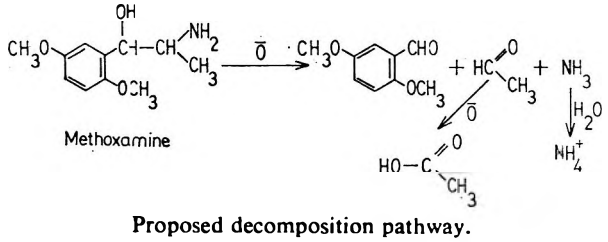


FIG. 4. Gas chromatographic separation of an aqueous sample of degraded methoxamine.

Peak B has the same retention time as acetic acid, a mixed sample showed only one peak. Peak A was due to 2,5-dimethoxybenzaldehyde and Peak C (programming at 4°/min) due to undegraded methoxamine. Due to high bleed, the column could not be used satisfactorily in the g.l.c.-m.s. system.

DISCUSSION

In aqueous solution, methoxamine hydrochloride is degraded by atmospheric oxygen to 2,5-dimethoxybenzaldehyde; this product was identified using a combination of the gas chromatograph and the mass spectrometer. The suggested oxidation pathway is shown below.



Proposed decomposition pathway.

The nmr spectrum of 2,5-dimethoxybenzaldehyde was recorded to confirm identification.

Acknowledgements

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Structural differences in solutions derived from polymorphic modifications of aspirin

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Differences in the structure of solutions derived from two polymorphic modifications of aspirin were demonstrated through differences in apparent pK_a values. The apparent pK_a's were determined in dimethylformamide using tetrabutyl-ammonium hydroxide as the titrant. The pK_a differences were ascribed to differences in intra- and intermolecular hydrogen bonding of the solute.

While polymorphic modifications of a substance have the same chemical properties, they may have different physical properties. It has been generally accepted that once in solution, all polymorphic modifications are identical; that is, there is no structuring of the solute that can be related to the original structure of the polymorph. However, solutes such as benzoic acid and salicylic acid are known to associate in certain solvents through hydrogen bonding, as do some solvents themselves. It is not surprising therefore that Urazovskii and co-workers (Urazovskii & Chetaev, 1949; Urazovskii & Kogan, 1950; Urazovskii, Kotlyarenko & Kuris'ko, 1959) were able to show differences in solutions derived from different polymorphic modifications. The present report utilizes similar methods to demonstrate structural differences in solutions derived from polymorphic modifications of apirin. The polymorphs are those whose dissolution properties from single crystals (Tawashi, 1968) and thermal properties (Tawashi, 1969; Summers, Carless & Enever, 1970) have recently been reported.

METHODS

Two polymorphic modifications of aspirin were prepared as described by Tawashi (1968). Polymorph I was prepared by slow crystallization at room temperature from a saturated solution of aspirin U.S.P. in 95% ethanol. Polymorph II was prepared by crystallization from a saturated solution of aspirin U.S.P. in n-hexane at room temperature.

The polymorphs were titrated in dimethylformamide using 0·1N tetrabutylammonium hydroxide in methanol-benzene as the titrant. A glass-modified calomel electrode system containing a saturated solution of potassium chloride in anhydrous methanol was used. Micro melting points were determined using a Mettler FP-2 hot stage.

RESULTS AND DISCUSSION

The two polymorphic forms of aspirin had melting points of 140° to 142° for polymorph I and 121·5° to 124° for polymorph II. The structural energy of the polymorph is reflected in the melting point, the intermolecular forces of the lower melting polymorph being of lesser magnitude than the forces present in the higher melting

polymorph. In aspirin it is possible to account for the difference in melting points by considering the effect of intra- and inter-molecular hydrogen bonding. Wheatly (1964) has shown that an aspirin structure crystallized from benzene and melting at 143° has a dimeric structure in the solid state. This is most likely polymorph I. Polymorph II would then be expected to have a lesser degree of intermolecular hydrogen bonding or increased intramolecular hydrogen bonding which is possible between the ester carbonyl and the hydroxyl hydrogen.

The solvation of two such polymorphic structures in the dissolution process could lead to solutions differing in acidic properties, provided the structures of the polymorphs were maintained on a molecular level during solvation and dissolution. That the solutions obtained from the two polymorphic forms of aspirin have different structural characteristics is demonstrated by the significant differences in apparent pK_a values obtained from these solutions. (Figs 1 and 2). Two structures for the

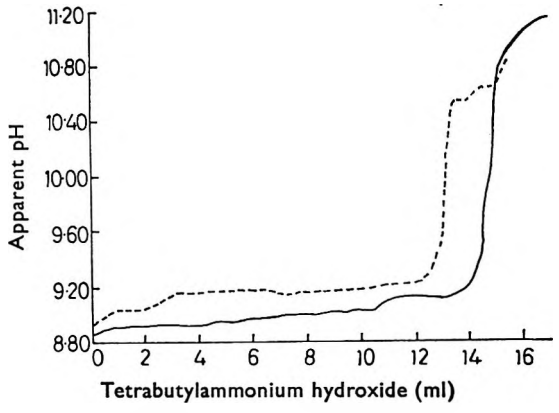


FIG. 1. Titration of aspirin polymorphs with 0·1N tetrabutylammonium hydroxide in dimethylformamide Polymorph I ——, II– – –.

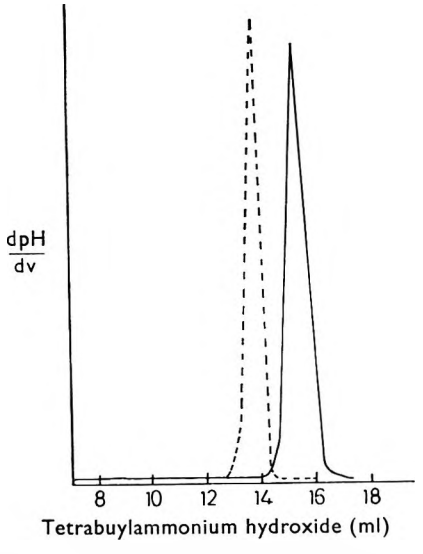
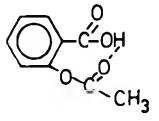
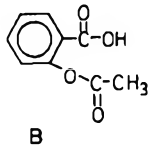


FIG. 2. First derivative plot of the titration of aspirin polymorphs. Polymorph I——, II – – –.

solute, aspirin, in solution are postulated to account for these differences in apparent pK_a (Structures A and B). Based on these two structures, it would be expected that structure A would have the highest pK_a value, and extrapolating to the solid polymorph, the lowest melting point because of the intramolecular hydrogen bonding. This is indeed the case of polymorph II, having a melting point of 121° to 124° is the weaker acid having an apparent pK_a of 9·19 in this solvent.



A



B

Polymorph I would be expected to be a stronger acid if its solvated structure involves little intramolecular hydrogen bonding, even if some dimer or higher forms exist in solution. In his *X*-ray analysis, Wheatly (1964) detected a great deal of angular distortion centered about the carboxyl and acetyl oxygen attached to the ring. Because of these stresses in the dimeric structure of the solid and because of the lower pK_a value obtained for polymorph I, it would appear that the solvated structure of this polymorph should be represented by structure B. Polymorph I was found to have an apparent pK_a of 8·99 representing a pK_a difference of 0·20. This is consistent with the above theory.

CONCLUSIONS

The concept of structured solutions resulting from polymorphic modifications could have profound implications in considerations of drug-receptor interactions and in drug availability from a dosage form in general. Many of the steroids, capable of existing in a large number of polymorphic forms under conditions of standard temperature and pressure, may be capable of forming structured solutions due to the presence of hydroxyl and carbonyl groups. These groups are most likely responsible for many of the polymorphic modifications. Differences in the properties of these and other polymorphic materials, as affected by the structure of their solutions, are being investigated further.

Acknowledgement

The authors are grateful to Professor Tawashi for a sample of the polymorph crystallized from n-hexane and for discussions with him concerning the preparation of this polymorph.

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LETTERS TO THE EDITOR

A reduced rate of turnover of brain noradrenaline during pentobarbitone anaesthesia

When using the specific tyrosine hydroxylase inhibitor, α-methyltyrosine methylester (H44/68) in the barbiturate-anaesthetized rat, Corrodi, Fuxe & Hökfelt (1966) demonstrated a reduced turnover of dopamine in the brain. However, the effect on brain noradrenaline was small, if any.

Brain noradrenaline disappears about twice as rapidly after dopamine β-hydroxylase inhibition as after inhibition of tyrosine hydroxylase (Goldstein & Nakajima, 1968; Bapna, Neff & Costa, 1970; Corrodi, Fuxe & others, 1970; Persson & Waldeck, 1970a). This observation as well as others led to a suggestion that there was an interaction between dopamine- and noradrenaline-containing neurons in the brain (Persson & Waldeck, 1970b). We therefore found it necessary to reinvestigate the effect of barbiturates on the rate of disappearance of noradrenaline after inhibition of its synthesis using H44/68 or the specific inhibitor of dopamine β-hydroxylase, bis(4-methyl-1-homopiperazinylthiocarbonyl)-disulphide (FLA-63) (Florvall & Corrodi, 1970, Svensson & Waldeck, 1969). This inhibitor appears not to have amine-releasing properties of its own (Andén & Fuxe: unpublished observations).

Female mice, about 20 g, kept at an ambient temperature of 30° were randomly grouped six by six. Pentobarbitone sodium (60 mg/kg, i.p.) was given alone or followed 15 min later by either H44/68 (200 mg/kg, i.p.) or FLA-63 (40 mg/kg, i.p.). Animals receiving either of the inhibitors alone were run in parallel, with untreated mice as controls. The animals were decapitated 2 h after the inhibitor had been given or 2 h 15 min after pentobarbitone. Noradrenaline in the brain was measured according to Bertler, Carlsson & Rosengren (1958).

Since 60 mg/kg of pentobarbitone was not sufficient to keep the animals sedated throughout the experiment, another dose regimen was introduced in the subsequent

Table 1. *The effect of pentobarbitone on the disappearance of noradrenaline from the mouse brain after inhibition of its synthesis.* Pentobarbitone was given either alone or 15 min before an injection of an inhibitor of tyrosine hydroxylase (H44/68) or of dopamine β-hydroxylase (FLA-63). In some experiments repeated doses of pentobarbitone were given. Mice receiving either of the inhibitors alone were run in parallel. Untreated animals served as controls. Two h after the synthesis inhibitor had been given, or 2 h 15 min after pentobarbitone administration, the animals were killed and noradrenaline in the brain determined. For time and dose schedules see text. Shown are the means ± s.e. in μg/g of three experimental groups each comprising 6 animals.

Pentobarbitone dosage mg/kg	Control	Pentobarbitone	H44/68	Pentobarbitone +H44/68	FLA-63	Pentobarbitone +FLA-63
60	0·52 ±0·047	0·47 ±0·026	0·32 ±0·009	0·31 ±0·012	0·19 ±0·006	0·22 ±0·009
80+20+20	0·53 ±0·047	0·58 ±0·040	0·34 ±0·031	0·38 ±0·023	0·18 ±0·007	0·34* ±0·024
80+20+20+20	0·48 ±0·032	0·56 ±0·019	0·38 ±0·038	0·35 ±0·107	0·18 ±0·013	0·38** ±0·017

* $P < 0{\cdot}01$. ** $P < 0{\cdot}001$ with respect to FLA-63 alone.

two experiments. An initial dose of 80 mg/kg was followed either by two further injections of 20 mg/kg given at intervals of 45 min, or three injections of 20 mg/kg given at intervals of 30 min. In these two experiments, animals receiving the synthesis inhibitors alone were given saline intraperitoneally to compensate for the possible dilution of the inhibitor caused by the repeated injections of pentobarbitone. Only the last mentioned dose regimen heavily sedated the animals throughout the experiment.

As in previous experiments, noradrenaline disappeared twice as rapidly after FLA-63 as after H44/68 (Persson & Waldeck, 1970a) (Table 1). Pentobarbitone, given alone, had no significant effect on brain noradrenaline. Nor did it change the rate of disappearance of the amine after H44/68 in any of the experiments. In a single dose, it also failed to change the rate of disappearance of noradrenaline after FLA-63. When two or three repeated injections of pentobarbitone were given, however, the decrease in noradrenaline brought about by FLA-63 was markedly inhibited (P <0·01 and 0·001 respectively).

H44/68 and FLA-63 in the doses used appear to inhibit the synthesis of noradrenaline equally well (cf. Svensson & Waldeck, 1969, 1971). Thus, the differences in the rate of disappearance of noradrenaline observed after the respective inhibitors may reflect differences in the level of activity of the noradrenaline-containing neurons, indicating an interaction between dopamine and noradrenaline-containing neurons in the brain (Persson & Waldeck, 1970b).

During pentobarbitone anaesthesia the activity of the dopamine-containing neurons is reduced as revealed by H44/68 (Corrodi & others, 1966). Does this reduction cause a change in the activity of the noradrenaline-containing neurons according to the interaction hypothesis? Using H44/68 this may be masked by the depletion of dopamine brought about by H44/68, thereby causing an impaired transmission. With FLA-63, which leaves the dopamine intact, a decreased rate of disappearance of noradrenaline after pentobarbitone was observed. This effect was more pronounced under a deep rather than a light anaesthesia.

It thus appears that during pentobarbitone anaesthesia the level of activity not only of the dopamine-containing but also of the noradrenaline-containing neurons is diminished.

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February 3, 1971

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On the positive inotropic action of glucagon in the isolated atria of the guinea-pig

Glucagon is known to exert a positive inotropic action in laboratory animals and in man (Klein, Morch & Mahon, 1968; Linhart, Barold & others, 1968; Lucchesi, 1968; Parmley, Matloff & Sonnenblick, 1969). However, the nature of the positive inotropic effect remains virtually unknown. It is probably different from that of cardiac glycosides (Parmley & others, 1969) and glucagon is not a sympathomimetic agent (Glick, Parmley & others, 1968; Mayer, Namm & Rice, 1970). Glucagon activates cardiac adenyl cyclase (Entman, Levey & Epstein, 1969; Mayer & others, 1970) but there is no conclusive evidence for a correlation between biochemical and positive inotropic effects. We therefore attempted to investigate the mechanism of action more closely.

Electrically stimulated, isolated atria (frequency 180/min) obtained from guinea-pigs, 280–420 g, of either sex, were suspended in oxygenated Muralt-Tyrode solution at 30°. Mechanical activity was recorded by means of a strain gauge and Helcoscriptor HE 86 t device. After 2 h of equilibration glucagon was added to the bath. The maximum increase in contractile force was approximately 20% of the initial value at a final glucagon concentration of 10^{-7}M. The effect was dose-dependent. The maximum effect was much lower than that of cardiac glycosides or sympathomimetic agents. Glucagon showed the same effect on the atria of animals pretreated with reserpine (3 mg/kg intraperitoneally for 2 days).

Pretreatment of normal atria with 10^{-7}M propranolol did not diminish the positive inotropic effect of glucagon (cf. Glick & others, 1966; Mayer & others, 1970). If glucose in the medium was replaced by sodium pyruvate (10 mM) the atria did not show a decrease in contractile force or frequency when compared with atria incubated in glucose-containing Tyrode solution. The tissue glycogen will have disappeared largely after incubation in a glucose-free medium (cf. Willebrands and van der Veen, 1967). The effect of glucagon (10^{-7}M) was the same in both media. Hence, an increased glucose concentration cannot explain the inotropic action. *N*-Isopropylmethoxamine (IMA) blocks the noradrenaline-induced rise in cyclic 3 : 5′-AMP (Shanfeld, Frazer & Hess, 1969). IMA (10^{-5} g/ml) added to the medium did not diminish the positive inotropic action of 10^{-7}M glucagon. Thus, a rise in 3,5′-AMP cannot be held responsible for the effect of glucagon.

Exposure of guinea-pig atria to 10^{-7}M glucagon (for 60 min) did not affect the sodium and potassium contents of the muscle preparations, nor were the membrane potential and the shape and size of the action potential changed. Obviously, glucagon does not give rise to changes of the cell membrane's properties which might explain the inotropic effect.

In separate studies we established the influence of glucagon (10^{-7}M) on the exchange of extracellular $^{45}Ca^{2+}$ against tissue Ca^{2+} in isolated atria (cf. Lahrtz, Lüllmann & van Zwieten, 1967).

Since the total calcium content was not affected by the presence of 10^{-7}M glucagon, the value shown for the total calcium concentration in Fig. 1 represents the mean value ($\pm$ s.e.) for both control organs and for atria treated with glucagon. Since neither $^{45}Ca^{2+}$-uptake nor the total calcium content were affected (Fig. 1), the exchangeable calcium fraction was not influenced either. The effect of 10^{-7}M glucagon on calcium metabolism was also studied in isolated atria that had been rendered failing upon equilibration in Tyrode solution for 12 h. The contractile force of these organs was much reduced in comparison with that of atria, equilibrated for only 2 h. The $^{45}Ca^{2+}$ content of the failing organs after either 15 or 60 min of incubation was not affected by 10^{-7}M glucagon in the medium. Accordingly, changes in Ca^{2+}

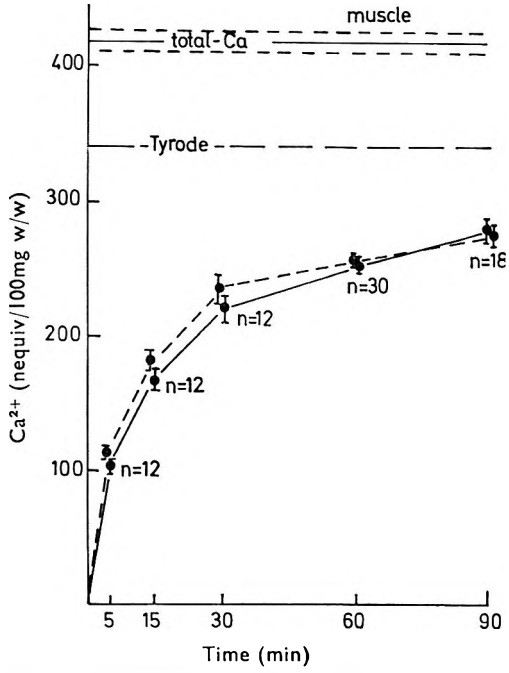

FIG. 1. Influence of glucagon (10^{-7}M) on calcium exchange in guinea pig isolated atria. The exchange, determined by means of $^{45}Ca^{2+}$, was expressed as nequiv Ca^{2+} per 100 mg wet weight. Each point on the curves represents the mean value ± s.e. for 12–30 atria. The total calcium content for both series of atria (glucagon and controls) is shown.

fluxes cannot be held responsible for the drug's inotropic effect.

Our results are in disagreement with those recently reported by Nayler, McInnes & others, (1970), who observed an increased $^{45}Ca^{2+}$ uptake by dog papillary muscle preparations upon treatment with glucagon. Since in these studies total calcium was not determined, the increased $^{45}Ca^{2+}$ uptake might as well be explained by means of changes in total calcium.

Glucagon influences calcium transport in isolated *subcellular* particles (Entman & others, 1969). However, it seems doubtful whether such observations will allow conclusions on the nature of the drug's effects in intact organs.

The present studies would suggest that none of the so-far known mechanisms underlying the positive inotropic action of drugs like cardiac glycosides, sympathomimetic agents, methylxanthines or Ca^{2+}-ions may explain the cardiac effect of glucagon. The nature of its positive inotropic action, therefore, requires further investigations.

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In vivo electrically evoked release of [^{3}H]noradrenaline from cat brain

A neurotransmitter role has been postulated for brain noradrenaline and dopamine. Before a substance can be considered to be a transmitter it must be shown to be released from nerve endings upon depolarizing stimulation. Baldessarini & Kopin (1966) demonstrated that electrical stimulation of brain slices caused the efflux of [^{3}H]noradrenaline, presumably from depolarized nerve endings. Attempts have also been made to detect endogenous dopamine release *in vivo* after electrical stimulation of various brain regions (McLennan, 1964), but the minute amounts of dopamine released were at the limits of the spectrophotofluorometric measurement. Philippu, Heyd & Burger (1970) reported that after the intraventricular injection of [^{14}C] noradrenaline, stimulation of the hypothalamus increased the concentration of ^{14}C (noradrenaline and metabolites) in ventricular perfusates. We now report an increased outflow of [^{3}H]noradrenaline into the ventricular system after electrical stimulation of the caudate nucleus.

Cats (2–3 kg) had their spinal cords sectioned and prepared for cerebroventricular perfusion (Carr & Moore, 1970). Five μCi of [^{3}H]noradrenaline (8·76 Ci/mmol, New England Nuclear Corp.) were injected in an effective volume of 10 μl through a cannula in a lateral ventricle at 16·5 A, 3·5 L (left or right) and + 8·0 D (Snider & Niemer, 1961). After 1 h the ventricular system was perfused with artificial cerebrospinal fluid (Pappenheimer, Heisey & others, 1962) at a rate of 0·1 ml/min. After washout for 110 min, the perfusion rate was increased to 0·5 ml/min and the collection of 1 ml samples of perfusate every 2 min was begun. During one or two of the collection periods, constant current square waves of 1 ms duration, 350 μA intensity and various frequencies were applied to the caudate nucleus by an electrode pair (anode at 13·0 A, 4·0 L and +5·0 D, cathode at 18·0 A, 4·0 L and +5·0 D). [^{3}H]Noradrenaline and metabolites in the perfusates were separated by alumina absorption and ion-exchange chromatography and quantified by liquid scintillation spectrometry (Carr & Moore, 1970). Throughout the course of the experiments blood pressure was recorded from the femoral artery and the rectal temperature monitored and maintained at 37·5° with a heating pad. All cannula and electrode placements were verified by gross dissection of the cat brain after formalin fixation.

In four experiments the mean ($\pm$ s.e.) concentrations of [^{3}H]noradrenaline and [^{3}H]normetanephrine in the perfusate samples before stimulation were 4·6 $\pm$ 1·1 and 2·6 $\pm$ 0·7 nCi/ml respectively. Electrical stimulation for 2 min significantly increased ($P < 0.05$) the perfusate concentrations in the periods during and immediately after the stimulation period (7·4 $\pm$ 1·1); stimulation did not alter the perfusate concentration of [^{3}H]normetanephrine. The other metabolites, deaminated catechols and deaminated *O*-methylated products, were present in the perfusates (10 and 15% of total radioactivity respectively), but they did not increase in concentration during or after stimulation.

When the effects of varying the stimulation frequency from 12·5–100 Hz upon the release of [^{3}H]noradrenaline from the caudate nucleus were examined, it was found that the initial period of stimulation at all frequencies tested in four experiments caused a significant increase ($P < 0.05$) in [^{3}H]noradrenaline perfusate concentration with the greatest release occurring at 50 Hz [increased release of [^{3}H]noradrenaline (nCi/ml) at 12·5 Hz = 0·8 $\pm$ 0·4, 25 Hz = 3·7 $\pm$ 1·6, 50 Hz = 6 $\pm$ 2·1, 100 Hz = 4 $\pm$ 0·7].

These facts support the idea of neurotransmitter roles for brain catecholamines by demonstrating depolarization-evoked release of [^{3}H]noradrenaline; the release is frequency-related, suggesting that it is related to neuronal function.

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Inhibitory action of γ-aminobutyric acid on cryoepilepsy in the frog

The inhibitory action of α-aminobutyric acid (GABA) in the central nervous system synapses has led some authors to investigate its influence on experimental convulsions and correlate its brain content in the central nervous system with convulsive disorders observed (Meynert & Kaji, 1962; Wood & Watson, 1969; Saad, 1970).

We report the action of GABA on an epileptiform attack produced by sudden cooling of the spinal cord of the frog (cryoepilepsy) (Ozorio de Almeida, 1943) and the relation to its content in the nervous centres.

GABA (0·5–5·0 g/kg) was injected into the ventral lymphatic sac of the frog and 1–48 h later the spinal cord was isolated and plunged into a temperature-controlled cooled Ringer bath (Ozorio de Almeida, Moussatché & Vianna Dias, 1941). After the induced convulsive attack, the cords were weighed, homogenized in 1 ml of ice-cold Ringer and centrifuged 15 min at 0° and 15 000 *g*. Free GABA was quantitatively estimated in the supernatant fluid by the *Ascaris lumbricoides* muscle bioassay (Ash & Tucker, 1967, as modified by Moussatché & Cordeiro, unpublished).

The relation between dose, temperature and the inhibition of convulsions is seen in Table 1. Doses of GABA greater than 3·0 g/kg, injected 1–5 h previously, inhibited the convulsions completely when the spinal cord was cooled to 6°; all the controls convulsed. The per cent inhibition of the convulsions is related to the bath temperature (Moussatché & Cuadra, 1967). After 24 or 48 h cords from GABA-treated frogs showed respectively 50%, inhibition or no inhibition when plunged in a bath at 5–6°.

Table 1. *The relation between temperature to which the spinal cord of frogs was exposed and the inhibition by GABA of epileptiform convulsions.*

GABA g/kg	Bath temperature °C	Convulsions observed %
3·0	4–6	0 (4)
5·0	4–6	9·5 (21)
5·0	0–4	33·3 (3)
Controls	4–7	100·0 (20)

Figures in brackets are number of frogs.

Table 2. *Concentration level of GABA in the spinal cord of the frogs and inhibition of cryoepilepsy at 5–6° C.*

	Time elapsed after GABA injection (5 g/kg) 1 h	3–5 h	24 h	48 h	Controls
GABA content	6·4±1·8*	4·5±1·0	3·1±0·2	1·4±0·2	3·0±0·4
Inhibition of convulsions (%)	100†	85†	50	0	0

* Each figure is the mean and s.d. of 3 groups of 2 frogs each.
† Different from controls $P < 0·05$.

In spite of the high dose of GABA injected and inhibition of the convulsions, the frogs themselves did not show apparent behavioural changes and jumped normally and showed normal postural reflexes.

Table 2 shows the GABA content in the spinal cord of the frogs and the per cent inhibition of the convulsions at intervals after GABA injection.

The results suggest that there is some relation between the concentration of GABA in the spinal cord of the frogs and the per cent inhibition of cryoepilepsy. These results are further evidence of the pharmacological activity of this amino-acid in the central nervous system.

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Inhibition of choline acetyltransferase and hexobarbitone-metabolizing enzymes by naphthylvinyl pyridine analogues

Certain naphthylvinyl pyridine derivatives inhibit rat brain choline acetyltransferase (ChA) *in vitro* (Smith, Cavallito & Foldes, 1967; Cavallito, Yun & others, 1969). Of these, *trans*-4-(1-naphthylvinyl)pyridine HCl (4-NVP) is the most specific inhibitor of ChA. We have found that 4-NVP markedly potentiates the cholinesterase inhibitor physostigmine, instead of inhibiting its behavioral effects as might be expected (Goldberg, Sledge & others, 1970). On the basis of other drug interaction studies, we suspect that 4-NVP inhibited microsomal drug metabolizing enzymes as well as ChA. The present investigation examines whether 4-NVP, a potent inhibitor, *in vitro*, inhibits ChA *in vivo*, assesses the relative inhibitory effects on the microsomal drug metabolizing system for hexobarbitone, and also evaluates other isomers of NVP against both parameters. For these purposes, *trans*-isomers of 2-(1-naphthylvinyl)pyridine HCl (2-NVP) and 3-(1-naphthylvinyl)pyridine (3-NVP) were prepared and investigated.

ChA inhibition was measured, both *in vivo* and *in vitro*, in mouse brain (male albino, 20 g) according to McCaman & Hunt (1965). The hexobarbitone-metabolizing enzyme system (HMES) was studied *in vitro* using the 10 000 *g* supernatant fraction of rat liver homogenized in mannitol-sucrose-EDTA (225-75-0·1 mM, pH 7·4). Side-chain oxidation of hexobarbitone was estimated by substrate disappearance (Cooper & Brodie, 1955). Evidence for *in vivo* inhibition of HMES was obtained by measuring sleeping time in mice after hexobarbitone sodium (100 mg/kg, i.p.). Drugs or saline were given 30 min before the barbiturate.

Molar concentrations required to inhibit both enzymes *in vitro* by 50% (I50) were estimated graphically from the means of at least duplicate analyses using several inhibitor concentrations.

All isomers inhibited both enzymes *in vitro* (Table 1), although an I50 value for 3-NVP against ChA could not be obtained. 4-NVP was most potent against both enzymes, and was about 17 times more active against HMES than against ChA. No such correlation was observed with the other analogues. 3-NVP had one-sixth of the activity of 4-NVP on HMES, yet was about 1/100th as active against ChA. Conversely, 2-NVP had about one-half the activity of 4-NVP against ChA, yet was some 20 times weaker as an inhibitor of HMES.

4-NVP produced a dose-dependent inhibition of mouse brain ChA in the dose range of 25·0 to 100·0 mg/kg. Further inhibition was not obtained after higher doses. It also caused an inhibition which persisted for at least 8 h after 200·0 mg/kg with a peak 2 to 4 h after administration (Table 2). At this dose, signs of depression and ataxia were observed. The acute intraperitoneal LD50 for 4-NVP at 24 h was 337 (290–539) mg/kg.

The effects on the duration of hexobarbitone sleep after NVP analogues agree closely with *in vitro* inhibition of HMES (Table 3). With the lowest dose that caused

Table 1. In vitro *inhibition of choline acetyltransferase (ChA) and the hexobarbitone-metabolizing enzyme system (HMES)*. Control preparations had enzyme activities equivalent to 9·8 μmol/g h^{-1} of acetylcholine formed and 91 μ mol/g protein h^{-1} of hexobarbitone metabolized.

Compound	I50–ChA	I50–HMES
4–NVP	3×10^{-5}M	5×10^{-6}M
3–NVP	(30% at 10^{-3}M)	3×10^{-5}M
2–NVP	7×10^{-5}M	1×10^{-4}M

Table 2. In vivo *inhibition of ChA by* 4-NVP. Values given are for each group of 4–8 mice. Mean control activity of 11·1 ± 0·6 (s.e.) μmol/g h^{-1} of acetylcholine formed (n = 27).

Dose (mg/kg, i.p.)	Time of death (h)	% Inhibition of controls ± s.e.
12·5	1	2·3 ± 2·2
25·0	1	13·1 ± 3·6
50·0	1	35·7 ± 5·8
100·0	1	63·8 ± 2·3
200·0	1	65·4 ± 3·1
200·0	2	81·2 ± 4·6
200·0	4	83·0 ± 2·7
200·0	8	58·9 ± 1·1
200·0	24	0·0

Table 3. *Effects of NVP analogues on hexobarbitone sleeping time.* Mean sleeping time (min) for 6 groups of controls (10/group) was 31 ± 5 (s.e.). There were 20 mice/drug treatment and a cut-off time of 120 min was used.

Dose (mg/kg) i.p.	% Control sleeping time 4-NVP	3-NVP	2-NVP
2·5	112	—	—
5·0	158*	—	—
10·0	242*	—	—
25·0	354*	130	108
50·0	—	187*	118
100·0	—	200*	196*

* $P < 0·05$ compared with controls.

prolongation of sleep, 4-NVP was 10 times more potent than 3-NVP, which in turn was twice as potent as 2-NVP. It seems that hexobarbitone is potentiated *in vivo* by inhibition of its metabolism by NVP analogues. After parenteral administration, NVP analogues seem to be capable of entering the central nervous system where they cause a significant and prolonged inhibition of ChA. The I50 for 4-NVP in mouse brain was identical to that obtained in rat brain by Smith, Cavallito & Foldes (1967). A correlation between inhibition of ChA and HMES is not evident within this series of three compounds.

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Increase of blood-brain barrier permeability to catecholamines by dimethyl sulphoxide in the neonate chick

Parenteral adrenaline or noradrenaline, because they cross a permeable blood-brain barrier in young chicks, produce sustained roosting or sleeping behaviour (Key & Marley, 1962; Spooner, Mandell & others, 1968; Hanig & Seifter, 1968). This phenomenon disappears within 6–8 weeks as the various components of the central nervous system mature (Waelsch, 1955; Lajtha, 1957). Several years ago, Brink & Stein (1967) showed that dimethyl sulphoxide (DMSO) facilitated the entry of [^{14}C]pemoline into the brain of rats. Recently, De La Torre (1970) has shown that DMSO increases the relative penetration of L-dopa and 5-HTP across the blood-brain barrier of the rat.

DMSO is an industrial solvent with skin-penetrating effects, and the ability to carry into the body toxic substances that are normally excluded (Stoughton & Fritsch 1964; Kligman, 1965; Narula, 1967). Since DMSO has both aqueous and lipid solubility characteristics that allow it to penetrate the central nervous system easily, it was chosen as a prototype compound for study of blood-brain barrier biogenic amine interaction in the neonate chick. We observed that DMSO intensified roosting induced by adrenaline and noradrenaline in the neonate, and therefore sought to determine whether this was associated with increased penetration of catecholamines into chick brain (Hanig, Morrison, & Krop, 1970).

One-day-old chicks housed in a temperature-controlled brooder were given free access to food and water and used within one week. Adrenaline or noradrenaline was dissolved in 0·9% NaCl or 50% DMSO—0·9% NaCl and administered (5 mg/kg, i.v.) into the jugular vein. This corresponded to a dose of 2·75 g/kg of DMSO which is well below the single dose toxicity for various species described by Smith, Hadidian & Mason (1967). Chicks were decapitated 10 min after adrenaline administration, but 2 min after noradrenaline because of its rapid turnover in the brain. Both amines in whole brain were estimated by an automated fluorimetric procedure (Hanig, Morrison & Krop, 1969).

The grossly observable behaviour of DMSO alone in chicks was minimal except for a transitory arousal that lasted for several seconds after injection, whereas those receiving adrenaline or noradrenaline with DMSO exhibited a roosting response that was more intensified than that observed with the same dose of either amine alone. Preliminary chemical studies showed no difference in endogenous amine concentrations between animals treated with saline or those treated with DMSO alone (Hanig & others, 1970). Administration of adrenaline + DMSO gave a highly significant increase of 34·8% in concentrations of this amine in brain over controls receiving the same dose of adrenaline alone. Similarly, noradrenaline + DMSO treatment gave

Table 1. *Dimethyl sulphoxide (DMSO) increases penetration of adrenaline and noradrenaline across the blood brain barrier of the neonate chick.*

Treatment	Concentration of adrenaline or noradrenaline†	Change %	n
Adrenaline	0·381	—	16
Adrenaline + 50% DMSO	0·513*	+34·8	15
Noradrenaline	0·614	—	24
Noradrenaline + 50% DMSO	0·852*	+38·8	24

* Significantly different ($P < 0·001$) from adrenaline and noradrenaline treatments, respectively.
† Expressed as μg/g whole brain.

a highly significant increase of 38·8% in brain noradrenaline over those receiving only the amine (Table 1). Experiments still in progress indicate that DMSO plus dopamine intensify fixed staring and catatonia that occur after administration of this amine alone, although the associated amine brain levels have not yet been determined.

Results indicate that DMSO, a solvent having both polar and nonpolar characteristics, facilitates entry of adrenaline, noradrenaline and possibly dopamine across the blood-brain barrier. The occurrence of this phenomenon may, in part, be related to the ability of this solvent, acting as a carrier of biogenic amines, to traverse both aqueous and lipid phases or components of the barrier. These findings may have significance in conditions requiring brain amine replenishment.

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The involvement of plasma free fatty acids in (+)-amphetamine-induced hyperthermia in rats

Both hyperthermia and increased levels of plasma free fatty acids (FFA) are seen in animals after the administration of amphetamine. Gessa, Clay & Brodie (1969) attributed the hyperthermia in rats to a peripheral site of amphetamine's action, *viz.*, the increased plasma FFA. However, Hill & Horita (1970) reported hyperthermia in rabbits to be due to its central action. In mice, low doses of amphetamine cause *hypo*thermia and large doses *hyper*thermia (McCullough, Milberg & Robinson, 1970). The former is attributed to a central component and the latter to a peripheral component of amphetamine's activities. Since the question of the mechanism of amphetamine-induced hyperthermia is still unanswered, we now report that an increase of plasma FFA is not an integral part of the hyperthermic response.

Male Wistar rats (Harlan Industries, Indianapolis), 175–200 g were housed six per group in a cage (50 × 80 × 40 cm). After determining rectal temperatures with a thermistor probe (TRI-R), desipramine (10 mg/kg, i.p. of the salt) was administered, 15 min later (+) amphetamine (4·0 mg/kg, i.p. base), was administered. Rectal temperatures were read at 30 and 60 min after the drug was given. Orbital sinus

blood was obtained immediately after measurement of body temperatures. The serum was collected and frozen until assay. FFA was extracted by the method of Dole (1956) and determined by the copper-soap method (Duncombe, 1969).

The results are shown in Fig. 1. Amphetamine increased both body temperature and plasma FFA. Desipramine potentiated the hyperthermia observed after amphetamine. This confirmed previous findings (Jori & Garattini, 1965). Desipramine antagonized the increase of FFA normally found with amphetamine. Since the plasma FFA was not increased in rats pretreated with desipramine, hyperthermia could occur without FFA release. This action of desipramine is not unexpected

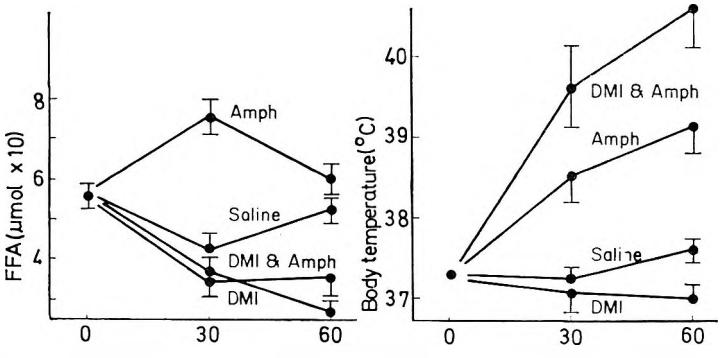

FIG. 1. Effect of desipramine on amphetamine-induced hyperthermia and plasma free fatty acid levels. DMI = desipramine. Amph = amphetamine. Values represent the mean ±s.e. from six rats.

since Finger & Page (1966) reported on its antilipolytic effects. Also, desipramine antagonizes the release of noradrenaline from adrenergic nerve endings by indirect-acting sympathomimetic amines (Brodie, Costa & others, 1968). We therefore have concluded that increased plasma levels of FFA are not required for the hyperthermic response to amphetamine. But we have not differentiated between a peripheral and central site of amphetamine's action.

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Genetic control of adjuvant-induced arthritis in rats

A widely used animal model of chronic inflammatory disease used for screening of anti-inflammatory or immunosuppressive effects of drugs, or both, is adjuvant-induced arthritis in rats (Pearson, 1956; Newbould, 1963; Andersen, 1970). However, the difficulty in evaluating these effects arises owing to the variation of manifest symptoms of the disease. Some experimental data suggest that the disease frequency might in part be dependent on genetic factors (Glen & Gray, 1965; Swingle, Jaques & Kvam, 1969). As this influence has not been studied systematically, we began an investigation of this aspect.

Several inbred strains of rats were tested (Table 1). Two of them (LEW and AVN) were chosen for further genetic analysis as there was a sharp difference in the incidence of the disease between them. A classical genetic method of analysis using both inbred strains, their first (F_1), second (F_2), and both backcross generations (B_1 and B_2), was employed. An estimate of the number of genes involved was based on testing the significance of differences between observed and expected disease frequency in all generations used, by use of a modified minimum χ^2-test (Elston, 1966).

Adjuvant arthritis was induced in 7–10 weeks old animals by an intradermal injection of 0·1 ml of complete Freund's adjuvant (5 mg of *Mycobacterium tuberculosis* vaccine/1 ml of mineral oil) into the left hind paw. Swelling of non-injected paws as the most prominent feature of the disease, was evaluated on the 21st day after the adjuvant administration.

Since there was no difference between females and males of either inbred strain (number of responding/total number: LEW: 9/9 and 10/10; AVN: 0/10 and 1/20, respectively), the pooled data of both sexes were analysed. The number of animals in

Table 1. *Incidence of adjuvant-induced arthritis in different inbred strains.*

	LEW	BP	Wistar	BN	AVN
Number of examined	19	5	10	5	30
Number of responding	19	2	3	1	1

Table 2. *Incidence of adjuvant-induced arthritis in different generations derived.*

	P_1 (LEW)	B_1 ($P_1 \times F_1$)	F_1 ($P_1 \times P_2$)	F_2 ($F_1 \times F_1$)	B_2 ($P_2 \times F_1$)	P_2 (AVN)
Number of examined ..	19	30	34	28	30	30
Number of responding ..	19	20	6	10	6	1
Probability of incidence ..	0·9794	0·6003	0·2212	0·3655	0·1306	0·0400
Observed incidence ..	100%	67%	18%	36%	20%	3%

all generations together with observed percentage of disease incidence and its probability, are given in Table 2. As the value of the F_1 generation shows, a higher resistance to induction of adjuvant disease is a dominant character. The measure of dominance, D, estimated according to Bruell (1962), reached the value of 0·65 indicating a partial dominance. The value of χ^2-test of deviations of observed frequencies of arthritis from expected ones was found to be 2·05 which is not statistically significant ($P > 0{\cdot}50$, d.f. = 3). Therefore, comparing this result with the frequency of the disease in other inbred strains, which suggests a polygenic control,

it is obvious that the polygenic system involved is of an additive character. The variation of the disease incidence should be, therefore, according to Grüneberg (1952) considered as a "quasi-continuous" one.

We reported here a so far not described case of nearly zero frequency of adjuvant-induced arthritis in the AVN strain of rats. On the other hand, there exists a 100% incidence of the disease in the LEW strain. These strains could be a suitable material for investigation of detailed pathogenesis of the disease.

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An apparatus for facilitating the measurement of tracer movements in a large number of muscle strips

Studies of the efflux of radioactive tracers from muscle have assisted the understanding of processes involved in contraction (Langer, 1968; Shanes & Bianchi, 1960; Van Breeman, 1969). The time course of tracer release is usually measured at convenient time intervals by assay of the medium in which the tissue has been suspended (Burgen & Spero, 1968). Should the specific activity of the tracer be low, the radioactivity released may very rapidly fall below the limits of detection, and it becomes necessary to assay the tracer remaining in the muscle strips as a function of time. Since it is frequently desirable to study tracer efflux under conditions similar to those under which contractility is measured, the strips should be set up under tension. The apparatus described below enables up to twenty-five tissues to be suspended under a tension of approximately 0·5 g in a single organ bath. The tissues may be removed rapidly for assay.

The tissues are suspended between small stainless-steel hooks which fit over wire projections from the base of a support, and stainless-steel springs attached to small pegs, which slot into the upper platform of the support, where they are retained by a low raised lip. The apparatus, (Fig. 1A) is placed in a 600 ml beaker the bath volume of which is effectively 400 ml and the physiological solutions are admitted to, and drained from, the bath under pressure. The solution is gassed down the central column of the tissue support, and the temperature is maintained by allowing the organ bath to stand in a temperature-controlled water bath.

In a typical experiment equilibrium is attained after 1 h in a suitable physiological solution. The bath is then drained and filled with medium containing the radioactive tracer, and after the required pre-incubation period, the bath is again drained, and the tissues are washed thereafter at frequent intervals, with fresh physiological solution. At convenient intervals one or more tissues are removed from the support and assayed

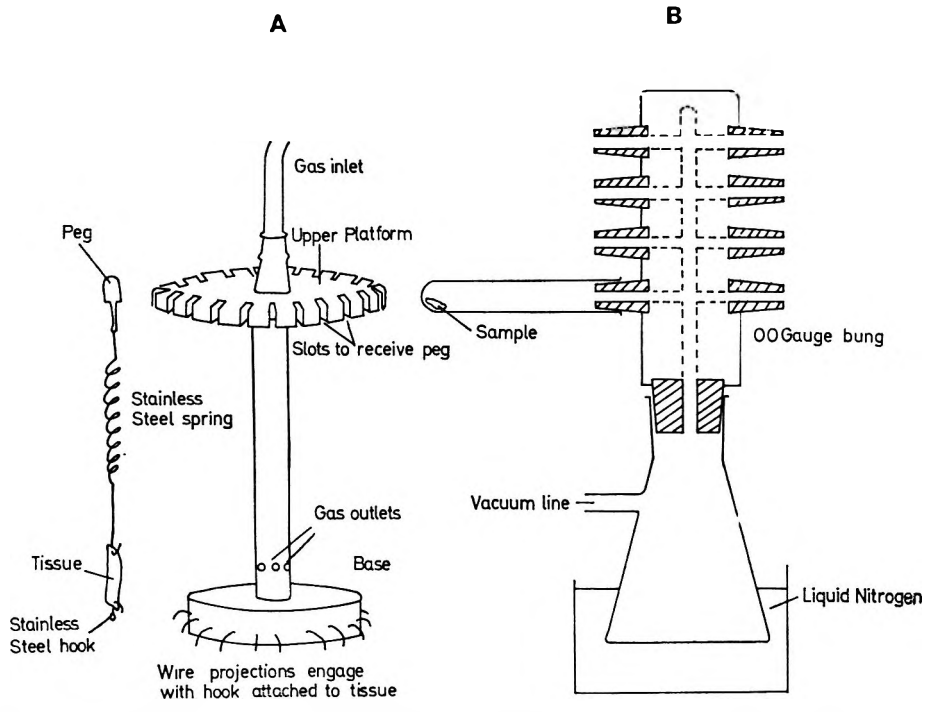

FIG. 1.A. Apparatus for the suspension of up to twenty-five tissues in a single organ bath. B. A simple apparatus for drying a large number of tissue samples.

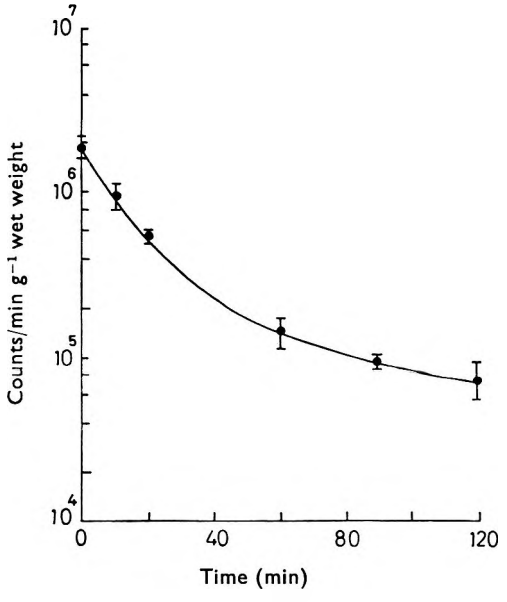

FIG. 2. The efflux of [^{3}H]digitoxin from rabbit myometrium using the apparatus shown in Fig. 1A.

for radioactivity. The desaturation curve of [^{3}H]digitoxin from rabbit myometrium, obtained by this means, is shown in Fig. 2.

A necessary adjunct to this apparatus, if the radioactivity is to be measured as d/min mg^{-1} dry weight of tissue, is a means of drying a number of small tissue samples. A simple device for this purpose is shown in Fig. 1B. Heavy Perspex rod, about 5 cm in diameter is drilled radially with four rows of eight perforations, and these are connected by a central channel drilled along the long axis of the rod. At each of the outlets around the rod, a gauge 00 rubber stopper bored with a single hole is recessed into the rod and cemented in place, care being taken to ensure that the connection is airtight. The central channel terminates in a gauge 8 rubber stopper also bored with a single hole, and recessed and cemented into place. The samples to be dried are placed in ignition tubes, frozen in liquid nitrogen and pushed on to the projecting stoppers. All outlets must be stopped, if necessary with empty tubes. The large stopper in the base is then inserted into the neck of a buchner flask which is supported in a bath of liquid nitrogen. The side arm of the flask is connected to a vacuum line.

Drying to a constant weight in this apparatus is achieved within 6–8 h, and thin tissues dry much faster.

I thank Miss R. V. Murthy for performing the experiments using [^{3}H]digitoxin. The significance of these studies will be reported elsewhere (Daniel, E. E. and Murthy, R. V., to be published). I am grateful to the Medical Research Council of Canada for financial support of this work.

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